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285. STUDIES ON EXPERIMENTAL TEAT AND MAMMARY DEVELOPMENT AND LACTATION IN THE GOAT

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(With 1 Plate and 12 Text-figures)

PART I. EXPERIMENTS ON THE GROWTH OF THE TEAT AND MAMMARY GLAND

WHILE much information is now available regarding experimental mammary development in laboratory animals (1, 2, 3), it is a curious fact that very few studies have been made on the cow and goat, the two farm animals of pre-eminent importance as milk producers. The most extensive study on the goat of which we are aware is that of de Fremery (4), who observed growth of the udder in female, but not in male, goats, to a size comparable with that attained during pregnancy, under the sole influence of oestrogen, additional administration of progesterone being unnecessary. In most species hitherto studied, the rudimentary male mammary gland possesses a capacity for growth, in response to suitable hormonal stimulation, equal to that of the immature female gland, and the failure of de Fremery (4) to produce mammary development in male goats is of considerable interest, particularly since cases of gynaecomastia accompanied by milk secretion in the male goat have been reported from time to time (5, 6).

No studies of teat growth in the goat have come to our notice, though the normal growth of the mammary gland in the goat has been described by Turner & Gomez (7).

The present observations were made in the course of a study of the experimental induction of lactation in the male and virgin female goat, a preliminary account of part of which has already been given (8).

Methods

The experimental animals were normal virgin female and normal castrated male goats kept under good conditions of management. Each goat was weighed weekly and the lengths of its teats measured with calipers. The expression 'teat length' as used throughout this paper refers to the mean of the lengths of the two teats. Most of the males were killed at the end of the experiment, when the mammary glands were removed and studied as whole mounts and

also microscopically. After removal each gland was pinned out on a cork board, fixed in Bouin's fluid for 24 hr., thoroughly washed in running water and the strip of connective tissue containing the gland dissected from the skin. The gland was overstained in borax-carmine, differentiated in acid alcohol, taken up through the alcohols and cleared and preserved in cedar-wood oil. The mammary gland area was determined by tracing the outline of the gland from a photograph on to squared paper.

A small segment of each gland was removed for microscopic examination, embedded in paraffin, sectioned at 8μ, and stained with haematoxylin and eosin.

Table 1. *Relative teat growth in normal and castrated immature male goats*

Goat	Age at beginning of observations days	Age at end of observations days	Body weight at beginning of observations lb.	Body weight at end of observations lb.	Teat length at beginning of observations mm.	Teat length at end of observations mm.	Equilibrium constant*	Probable error†
			(*a*) Castrated males					
Darby	76	258	51	93¼	13·0	17·0	1·09	±0·13
Laurel	36	211	17	73	9·5	17·0	0·98	±0·07
Hardy	36	141	11½	36	7·0	11·0	1·06	±0·15
Punch	45	153	16	38	10·0	14·0	1·07	±0·09
William	66	174	33	55	12·0	15·0	0·92	±0·16
Mean equilibrium constant weighted according to probable errors†							1·02	±0·02 (r_e)‡
			(*b*) Normal males					
Darby (before castration)	27	69	21	46	11·0	12·0	0·59	±0·28
Pantagruel	2	211	9¼	74¾	7·0	13·0	0·87	±0·11
Timothy	46	151	25	63	7·5	13·0	1·35	±0·13
Mean equilibrium constant weighted according to probable errors†							1·04	±0·15 (r_e)‡

* The equilibrium constant (see Huxley & Teissier[12]) is obtained by logarithmic plotting of teat length against $\sqrt[3]{}$(body weight) and determination of the slope of the best fit straight line by the method of least squares. For isometric growth the equilibrium constant is unity (see Huxley[11]).

† See Bond[13]. ‡ r_e is the probable error calculated from residuals (see Bond[13]).

Teat growth in the castrated male

Since the experiments were carried out on young growing animals, it was necessary for a complete analysis of the growth phenomena to study not only changes in absolute teat length, but also in the rate of teat growth relative to the growth of the body as a whole. In order to assess the effect of the various experimental procedures a knowledge of the relative rate of teat growth in the untreated animals was therefore required. Throughout this paper the expression 'relative teat growth' means the rate of teat growth compared with the rate of general body growth instead of the usual meaning, rate of growth per unit size. Observations were made on five castrated males, particulars of which are given in Table 1. As in the case of studies on teat

growth in the guinea-pig(9) and mammary growth in the monkey(10), the cube root of the body weight was adopted as a reference standard, since the body-weight increase was considered to provide the most satisfactory all-round measure of general body growth. To test the agreement of the data for each goat with Huxley's(11) simple allometric law, $\log_{10}$ teat length was plotted against $\log_{10}$ body weight, and the slope of the best fit straight line calculated by the method of least squares. The equilibrium constants(12) so obtained and their probable errors (calculated by a method given by Bond(13)) are given in Table 1. It will be seen from the upper section of Table 1 that when the individual equilibrium constants were combined, each being weighted according to its probable error, the mean value, $1{\cdot}02 \pm 0{\cdot}02$, was approximately unity, the value required for isometric growth. It may therefore be concluded that in the young castrated male goat, teat growth is isometric over the range of age given in Table 1.

Teat growth in the normal male

The observations on normal males are rather scanty, on account of difficulties involved in keeping these animals. Only selected specimens required for stud purposes are normally kept, and in any event uncastrated male goats require special housing at a distance from the milking shed on account of their violent strength and strong smell during the rutting season. Nevertheless, observations were secured on three immature normal males (see lower section of Table 1). The equilibrium constants are rather variable and the probable errors large. The combined value for the equilibrium constant, $1{\cdot}04 \pm 0{\cdot}15$, however, allows of the tentative conclusion that teat growth in the immature normal male is approximately isometric.

Experimental growth of the teat and mammary gland in male goats

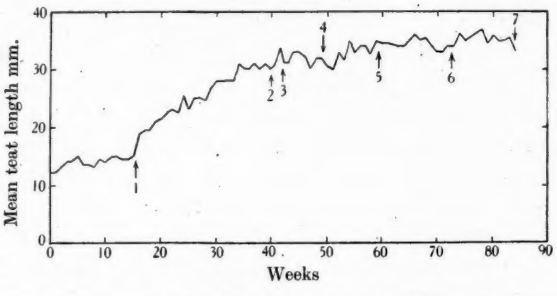
The oestrogen used throughout these experiments was diethylstilboestrol or its dipropionate. In most experiments diethylstilboestrol was administered by subcutaneous implantation of compressed tablets under local anaesthesia (Percaine), the most convenient technique for ensuring the prolonged action of physiological doses of most sex hormones(14). In two cases diethylstilboestrol dipropionate incorporated into an ointment was administered by inunction of the skin round the base of the teats. In one experiment progesterone (subcutaneously injected in oily solution) and in another, ethinyltestosterone (anhydro-hydroxy-progesterone) (implantation of tablet), were administered in addition to oestrogen.

The most prolonged experiment was made on a male goat, William, castrated at 63 days of age. A tablet of diethylstilboestrol weighing 960 mg. was subcutaneously implanted at 177 days of age and removed 169 days later because the relative rate of teat growth, which had notably increased shortly after implantation, had by then fallen off considerably, and it was thought that the tablet might have become encapsulated with fibrous tissue and

absorption thereby retarded (15). On removal it was in fact found that the tablet was encased in a sac of fibrous tissue from which a yellowish paste exuded when cut. Approximately 220 mg. had been absorbed from this tablet.

A fresh tablet weighing 964 mg. was immediately inserted, followed 16 days later by a tablet of ethinyltestosterone weighing 500 mg. Both tablets were removed after a further 51 days, by which time approximately 115 and 109 mg. had been respectively absorbed. The dual treatment had caused no further spurt in relative teat growth, nor was it accompanied by any signs of udder development. In fact, Text-fig. 1 indicates that the insertion of the tablet of ethinyltestosterone was followed by a slight regression in teat length, and further growth did not occur until after the tablet had been removed.

Seventy days after the removal of these tablets a further attempt to stimulate an increase in the relative rate of teat growth and also, it was hoped, udder development, was made. A sterile tablet of diethylstilboestrol

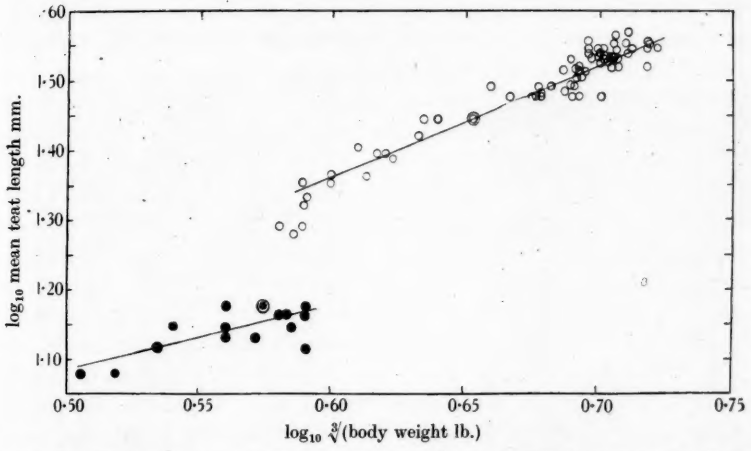


Text-fig. 1. Effect of treatment with diethylstilboestrol and ethinyltestosterone on teat growth in the castrated, immature male goat William. At 1, a 960 mg. tablet of diethylstilboestrol was subcutaneously implanted; at 2 it was replaced by a fresh one weighing 964 mg. 3 denotes the implantation of a 500 mg. tablet of ethinyltestosterone. Both tablets were removed at 4. A 971 mg. tablet of diethylstilboestrol was implanted at 5 but was extruded after 81 days. A fresh tablet weighing 416 mg. was inserted at 6. The goat was autopsied at 7.

weighing 971 mg. was implanted with aseptic technique in the hope that encapsulation of the tablet and consequent possible retardation of absorption would thereby be prevented or delayed. This tablet was in position for 81 days. For some days before the end of this period the existence of a scab at the site of implantation had been noticed, and it appeared that the tablet was in imminent danger of being extruded. The tablet was found to be missing on the morning of the 82nd day. A fresh sterile tablet weighing 416 mg. was aseptically implanted 14 days later and removed after 82 days when it had lost 52 mg. It is worth noting that this tablet was also found to be encapsulated on removal, as indeed happened in all cases throughout these experiments whether or not the tablet was sterilized and implanted with aseptic precautions.

Absolute teat lengths are plotted in Text-fig. 1 and relative teat lengths plotted logarithmically in Text-fig. 2. The general conclusion to be drawn

from both graphs is that the first implantation caused an immediate and appreciable increase in the rate of teat growth, which, however, progressively declined despite subsequent renewals, even when oestrogenic treatment was supplemented with ethinyltestosterone, a substance with the same physiological activity as progesterone. The points in Text-fig. 2 which fall within the period of treatment seem to lie on an asymptotic curve, which would indicate that during this period of allometric growth the equilibrium constant was progressively diminishing. It must be noted, however, that towards the end of this period the points are so crowded together, owing to the slowing up of body growth, as practically to defy analysis. It might with some justifi-



Text-fig. 2. Effect of treatment with diethylstilboestrol and ethinyltestosterone on relative teat growth in the castrated, immature male goat William. The black points relate to the period before treatment, the circles to the period during which diethylstilboestrol and ethinyltestosterone were administered by subcutaneous implantation of tablets (see text). Teat growth during the pre-treatment period was approximately isometric, since the equation of the best fit straight line is $y = 0{\cdot}92x + 0{\cdot}62$. Allometric growth occurred during the treatment period. The straight line shown, which is the best fit straight line for all but the first three circles, corresponds to the equation $y = 1{\cdot}61x + 0{\cdot}40$.

cation be maintained that all of the post-implantation points, except the first three, lie on a fair approximation to the straight line indicated in the figure. At any rate, at least over the mid-experimental period, the data agree approximately with the law of simple allometric growth, the equilibrium constant having the value of 1·6.

No external signs of udder development were observed throughout the experiment. The undissected whole mount of one of the glands removed at autopsy is shown in Pl. 1 *a* and should be compared with Pl. 1 *b*, which shows a whole mount of a gland taken from the untreated, castrated male goat, Hardy. It would appear that some alveolar development is present,

and this is confirmed by microscopic examination (Pl. 1 *c* and *d*). The area of the gland shown in Pl. 1 *a* was 2·5 cm.²

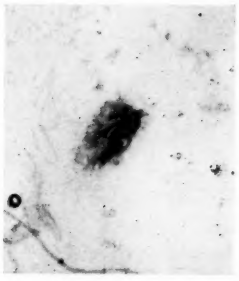
Tablets of diethylstilboestrol were subcutaneously implanted into two other male goats. A normal male, Timothy, received a sterile tablet weighing 739 mg. at 153 days of age. Forty-nine days later the tablet was observed to be on the point of working out and was removed. It was resterilized and aseptically replaced the same day. The goat carried this tablet for a further 177 days, at the end of which time he was killed and the tablet removed. In all, approximately 195 mg. were absorbed. Absolute teat-length curves show little evidence of a spurt of teat growth following either implantation; relative curves, however, indicate that there was some stimulation of relative growth. Again no udder growth was observed. The whole mounts (Pl. 1 *e*) point to the presence of alveoli, which is confirmed by microscopic examination (Pl. 1 *f*). The mean mammary gland area was 5·5 cm.²

The second goat, Laurel, was castrated when 33 days old, and a sterile tablet weighing 849 mg. aseptically implanted 181 days later. Sixteen days later this tablet was found to be missing and was replaced aseptically by another sterile tablet weighing 1020 mg. This tablet was removed 426 days later together with the right mammary gland. During this period the weight of the tablet had decreased by 394 mg. Again the absolute teat-growth curves showed no evidence of stimulation, but the relative curves indicated an increase in relative growth rate. Examination of the whole mount of the mammary gland removed at operation revealed a gland of 2·7 cm.² area and of appreciable thickness. The gland was so dense that examination of the undissected whole mount revealed no details of its structure. Microscopic examination of sections of this gland showed the presence of large ducts but no alveoli.

It will have been noted that in three cases, after subcutaneous implantation of diethylstilboestrol tablets, the small incision in the skin failed to heal satisfactorily and the tablet was eventually extruded. Experience showed that this happened when the length of the 'pouch' opened by blunt dissection between the skin and the subcutaneous tissues was not sufficient for the tablet, after insertion, to lie well away from the incision. When a sufficiently long pouch was made no such trouble was experienced.

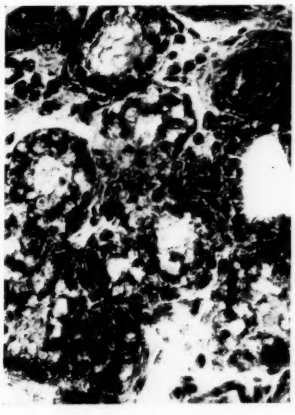
Two castrated males were used for experiments by inunction. A male goat, Punch, castrated at 42 days of age, was given a course of thrice-weekly inunctions with 1·0 g. of ointment containing diethylstilboestrol dipropionate, beginning when he was 156 days old. During the first 141 days of the inunction period 1% ointment was used. The strength of the ointment was increased to 2½% during the next 65 days, and over the final 31 days 5% ointment was used. During the last 32 days of the period of treatment with 2½% ointment and the whole of the period of treatment with 5% ointment, subcutaneous injections of 5 mg. progesterone in 1 ml. sesame oil were given three times weekly in the hope that the combined treatment would promote

(*a*) Whole mount of mammary gland of William, a castrated immature male goat which received prolonged treatment with diethylstilboestrol and ethinyltestosterone. ×2·2.

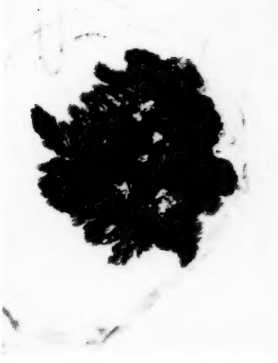


(*b*) Whole mount of mammary gland of Hardy, an untreated, castrated, immature male goat. ×3·2.

(*c*) Photomicrograph of part of the mammary gland of William, showing alveoli. ×118.



(*d*) High-power view of alveolar tissue in mammary gland of William. ×580.



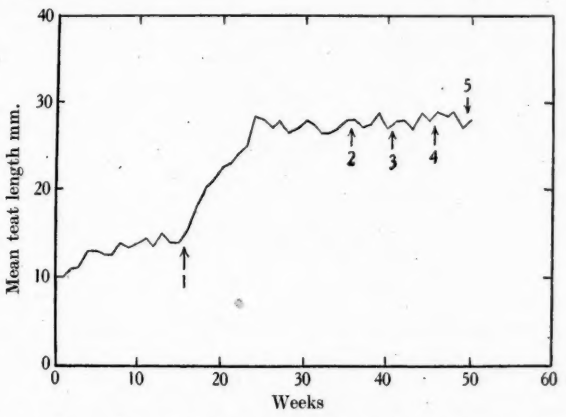
(*e*) Whole mount of mammary gland of Timothy, an immature normal male goat which received prolonged treatment with diethylstilboestrol. ×1·6.

(*f*) Photomicrograph of part of mammary gland of Timothy, showing alveoli. ×118.

udder growth. This, however, did not occur. The goat was killed 23 days later and the mammary glands removed.

The absolute and relative teat-growth curves for this goat are given in Text-figs. 3 and 4. The treatment with the smaller dose of oestrogen clearly caused a sudden change from isometric to allometric growth (equilibrium constant $= 3{\cdot}79 \pm 0{\cdot}38$), but when the teats had reached a mean length of about 28 mm. growth virtually ceased despite increase of the dose. Examination of the whole mounts of the mammary gland at the end of the experiment revealed the presence of nothing but a few ducts radiating from the base of the teat.

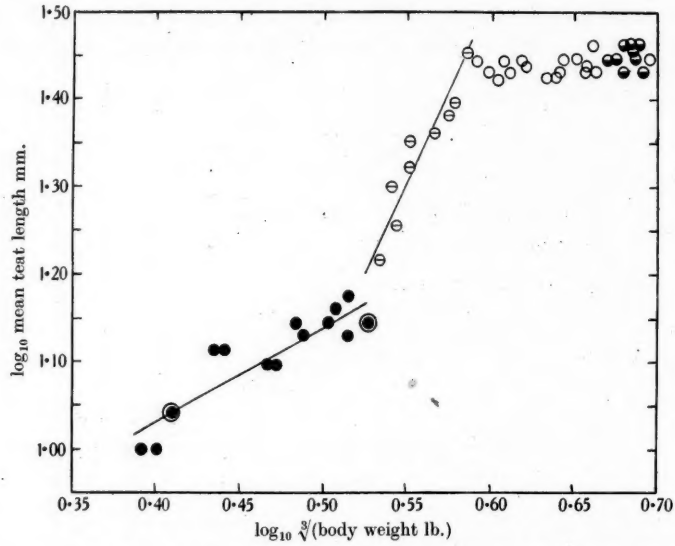
A castrated male goat, Gargantua, was used for a final attempt to produce udder development in the male. It was thought possible that the presence



Text-fig. 3. Effect of diethylstilboestrol dipropionate (applied by inunction) and progesterone on teat growth in the immature, castrated male goat Punch. At 1, thrice-weekly inunctions with 1·0 g. 1% diethylstilboestrol dipropionate ointment began; at 2 the strength of the ointment was increased to 2½%; at 3 thrice-weekly subcutaneous injections of 5 mg. progesterone began; at 4 the strength of the ointment was increased to 5%; at 5 treatment with diethylstilboestrol dipropionate and progesterone was discontinued.

of the active testis might exert some influence on the rudimentary gland which would render it permanently insensitive to oestrogen stimulation. The testes were therefore removed on the day following birth, and therefore presumably before they had begun to secrete androgen. A second possible reason for failure to promote experimental mammary development in males may have been that the doses of oestrogen previously used had been too high, thus leading to inhibition rather than stimulation of mammary growth (16, 17, 18). Accordingly, this goat was given a prolonged treatment with a low dose of diethylstilboestrol dipropionate, the dose being increased at intervals as the animal grew, so that the dose/kg. remained approximately constant. He received in the first instance three inunctions weekly with 1 g. of 0·1% diethylstilboestrol ointment over a period of 31 days beginning at 5 days of

age, and the strength of the ointment was increased by 0·1% as successive 10 lb. increments of body weight were reached, until a strength of 0·9% was attained, and he had been 278 days under treatment. During the first 112 days of the experimental period the teats grew allometrically (equilibrium constant = 2·03 ± 0·11) to a maximum length of about 20 mm. and then growth ceased. No udder development occurred. Since the results of 278 days of treatment as described were disappointing, it was decided before killing the goat to increase the dose of oestrogen considerably in case the doses previously used had been too low. He was therefore given inunctions thrice weekly with

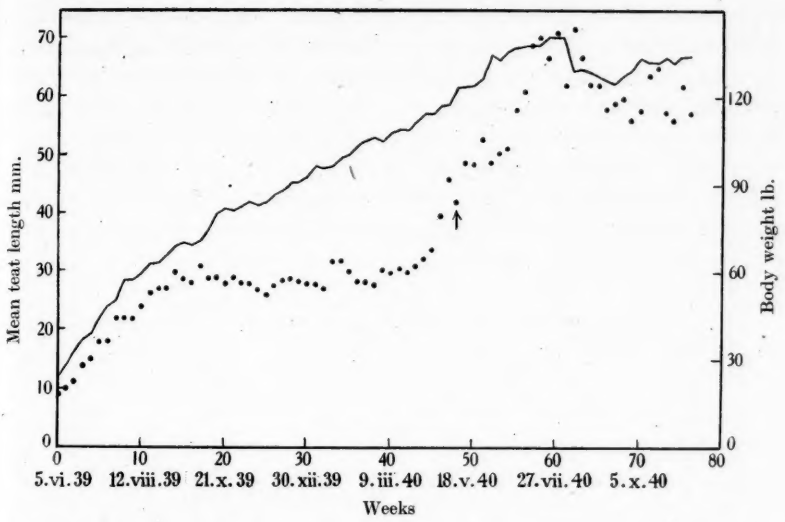


Text-fig. 4. Effect of diethylstilboestrol dipropionate (applied by inunction) and progesterone on relative teat growth in the castrated, immature male goat Punch. Observations made before treatment, shown as black points, show that teat growth was then approximately isometric, since these points are fitted by the straight line $y = 1{\cdot}07x + 0{\cdot}60$. Barred circles represent observations made during the first part of the inunction period when allometric teat growth occurred. The equation of the straight line which fits these points is $y = 3{\cdot}79x - 0{\cdot}78$. Half-black circles relate to the period during which progesterone was administered.

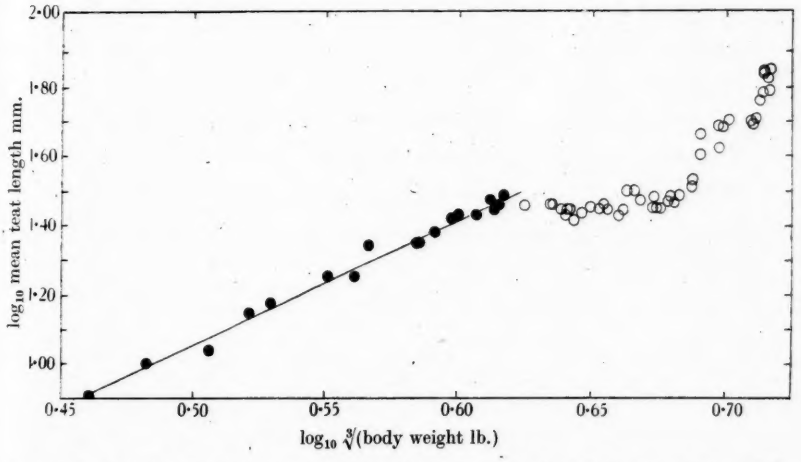
1·0 g. of 2·0% diethylstilboestrol ointment. After 68 days of this new treatment no further teat or udder growth was observed.

Teat growth in the normal female

Since we have reason to believe that the teat-growth response to oestrogens is extremely sensitive (19), it was hoped that teat-growth studies would indicate when the caprine ovary first begins to secrete oestrogen. The absolute and relative teat-growth curves for the virgin British Saanen goat Snowwhite (Text-figs. 5, 6) show that allometric growth was in progress as early as

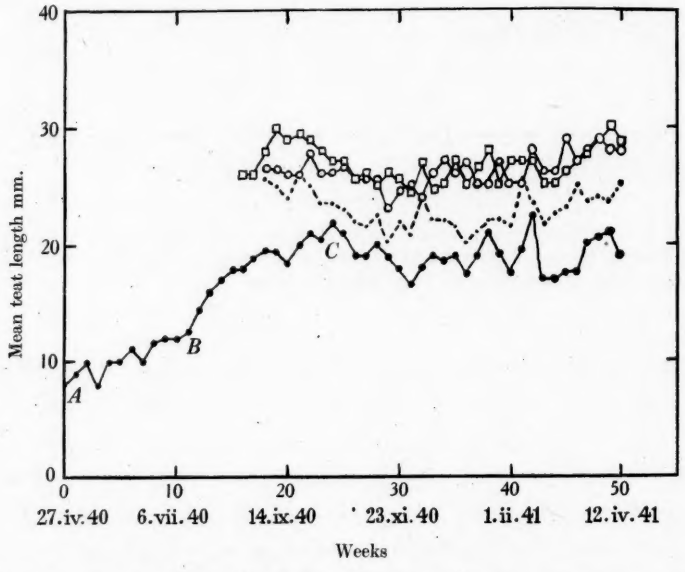


Text-fig. 5. Teat growth in the untreated virgin goat Snowwhite, showing cessation of teat growth during the breeding season. The dotted curve represents mean teat length and the unbroken curve, body weight. At ↑, daily milking began.



Text-fig. 6. Relative teat growth in the untreated virgin goat Snowwhite. Observations made between 41 days of age and the beginning of the breeding season following her birth are shown as black points. During this period the teats grew allometrically, the equation of the best fit straight line for the black points being $y = 3{\cdot}66x - 0{\cdot}78$. The circles represent observations made during her first breeding season, when teat growth ceased and during the subsequent anoestrous season when allometric growth was resumed.

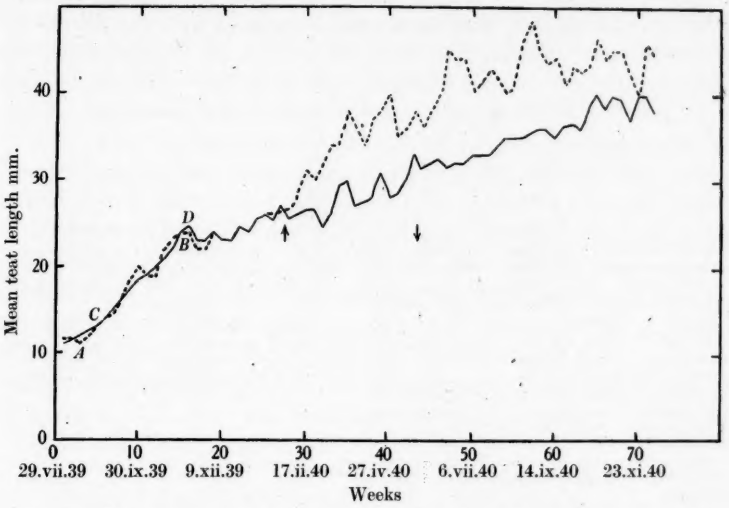
41 days after birth, when observations on this goat began. A point of great interest about the teat-growth data for this goat is that during the breeding season following her birth, teat growth ceased completely (Text-fig. 5) even though the goat was still growing, to be resumed allometrically during the next non-breeding season. During the first period of allometric growth, the data agreed excellently with the simple allometric law, the equilibrium constant being $3{\cdot}66 \pm 0{\cdot}07$. Data for four other young goats (see Text-fig. 7) also indicate the absence of teat growth during the breeding season. No data previous to about 5 months of age are available for three of these, since they were purchased at the beginning of their first breeding season at approxi-



Text-fig. 7. Teat growth in four immature British Saanen female goats, during the year following their birth, showing cessation of teat growth during the breeding season. In the case of Belinda (black dots), logarithmic plotting of the teat lengths against $\sqrt[3]{}$(body weight) shows that *AB* was a period of isometric growth and *BC* a period of allometric growth.

mately 5 months of age. From comparison with data obtained on goats of the same breed born at this Institute however, it may be concluded that previous to purchase the teats must have been growing allometrically to have attained the length in proportion to body size which they had at the time of purchase. The fourth of these four females, Belinda, was bred at this Institute, and it was possible to study the growth of her teats from birth. Her teats grew isometrically until she was 108 days old when positive allometric growth began. Endocrine activity evidently developed more slowly in her ovaries than in those of Snowwhite.

The data for two twin females (see Text-fig. 8) were not in accord with the aforementioned observations, since during the early part of their first breeding season the teats of both were growing allometrically. These goats were born in June, and it is probable that when the rutting season began in the following September, they had not attained sexual maturity and therefore



Text-fig. 8. Effect of diethylstilboestrol applied by inunction on teat growth in the virgin goat Poppy. The broken curve relates to the experimental goat Poppy and the unbroken curve to the twin control goat Pansy. ↑ denotes beginning of inunction three times weekly with 1·0 g. of 1% diethylstilboestrol ointment in the case of Poppy and 1·0 g. inactive ointment base in the case of Pansy. ↓ denotes the last inunction. Logarithmic plotting of the teat lengths against $\sqrt[3]{}$(body weight) shows that *AB* and *CD* were for Poppy and Pansy respectively periods of allometric teat growth.

Table 2. *Teat-growth data for normal virgin female goats*

Goat	Equilibrium constant	Probable error	Time of onset of teat allometry: age in days
Snowwhite	3·66 (allometric phase)	±0·07	<41
Poppy	3·45 (allometric phase)	±0·16	51
Pansy	1·10 (isometric phase)	±0·05	65
	3·12 (allometric phase)	±0·11	
Belinda	1·10 (isometric phase)	±0·13	108
	2·08 (allometric phase)	±0·14	

did not exhibit oestrous cycles; in other words, the breeding season, for them, began late.

The teat-growth curves for both of these females show discontinuities which correspond to the start of allometric teat growth and enable the age of onset of teat allometry to be estimated. These values, together with those for Snowwhite and Belinda, are given in Table 2.

It would thus appear that in the goat the ovary often begins to secrete sufficient oestrogen to cause allometric teat growth soon after birth, and that during the breeding season, when oestrous cycles are in progress, some factor operates to prevent teat growth.

Table 2 also gives the equilibrium constants and their probable errors for the allometric phase of teat growth in the four home-bred goats. It will be seen that in all cases the data show good agreement with the simple allometric law.

Table 3. *Experimental teat growth in the virgin female goat*

Goat	Details of treatment	Age when treatment began days	Result of treatment	Approx. maximum teat length attained mm.
Jemima	28. ix. 39, daily milking* began. 7. xi. 39–9. xii. 39, inunction of udder three times weekly with 1·0 g. base ointment. 12. xii. 39–20. viii. 40, inunction of udder three times weekly with 1·0 g. 1% diethylstilboestrol ointment	164	An increase in the rate of teat growth began on 23. iii. 40, i.e. 100 days after oestrogen treatment was initiated	44
Fanny	17. vii. 39–7. xi. 39, inunction of udder three times weekly with 1·0 g. 1% diethylstilboestrol dipropionate ointment. 22. vii. 39, daily milking began. 7. xi. 39, inunction of udder three times weekly with 1·0 g. inactive base ointment. 12. xii. 39, inunction of udder three times weekly with 2·0 g. 1% diethylstilboestrol dipropionate ointment. 30. i. 40–6. viii. 40, inunction of udder three times weekly with 1·0 g. 1% diethylstilboestrol dipropionate ointment	499	An increase in the rate of teat growth began on 18. v. 39, i.e. 33 days after oestrogen treatment was initiated	43
Poppy	30. i. 40–21. v. 40, inunction of udder three times weekly with 1·0 g. 1% diethylstilboestrol ointment. 31. i. 40 daily milking began	215	An increase in the rate of teat growth as compared with the control (Pansy) began on 10. xii. 40, i.e. 11 days after oestrogen treatment was initiated	45
Pansy	30. i. 40–21. v. 40, inunction of udder three times weekly with 1·0 g. inactive ointment base. 31. i. 40, daily milking began	215	No increase in rate of teat growth due to inunction with base ointment and milking	40
Jill	22. vii. 39, daily milking began. 10. x. 39–30. xi. 39, inunction of udder three times weekly with 1·0 g. base ointment. 30. xi. 39, inunction of udder three times weekly with 1·0 g. 1% diethylstilboestrol dipropionate ointment	362	An increase in the rate of teat growth began on 5. viii. 39, i.e. 14 days after the beginning of daily milking. Inunction with inactive ointment base and oestrogenic ointment caused no further increase in growth rate	37

* The word 'milking' is used in this table to denote an attempt to withdraw milk from the teats. It does not necessarily imply that the goat was giving milk (but see Part II).

Experimental teat and mammary growth in virgin females

Observations have been made on four virgin females which received courses of udder inunctions with diethylstilboestrol or its dipropionate. Particulars of these experiments are given in Table 3. Of the four experimental goats referred to in this table three showed an increase in the rate of teat growth at various times after the initiation of treatment, while in the fourth the oestrogen treatment had no apparent effect, though a small increase in the rate of teat growth occurred shortly after the initiation of attempts to draw milk from her teats. The response of the teat in the young female to oestrogen stimulation is particularly well shown in a well-controlled experiment in which

twin females (Poppy and Pansy) were used. They received identical treatment (see Table 3), save that the ointment used for one contained oestrogen while the other was treated with inactive ointment. The possible effects of daily milking and udder massage associated with inunction were therefore eliminated. The absolute teat-length curves are shown in Text-fig. 8. It is seen that the curves for both goats were practically identical until treatment began, shortly after which the teats of the oestrogen-treated goat began to grow faster than those of the control. The teats of the experimental goat reached a maximum length of about 45 mm., a length which was eventually approached by those of its twin.

Quantitative observations on udder development were not made on any of these females on account of the difficulty of making objective measurements of udder size. It was noted in most cases, however, that a considerable amount of udder growth occurred 3 or 4 weeks after application of diethylstilboestrol. In the experiment with twin virgin females, udder development set in much earlier in the experimental animal than in the control, and her udder attained larger dimensions, while in the case of Jill, daily attempts to obtain milk from her teats combined with inunction with inactive ointment base produced very little udder growth, which did, however, occur soon after oestrogenic ointment was applied. De Fremery's observations (4) on the stimulation of udder growth in the goat by oestrogen are therefore confirmed.

Discussion

Teat growth in the young castrated male goat is, as might be expected, isometric relative to general body growth, and our rather scanty observations would appear to indicate that the same is true of the normal male. In the goat, therefore, the testes do not seem to be responsible for maintaining isometric growth of the teat as is the case in the guinea-pig (9). Neither, according to the observations of Turner & Gomez (7), do the testes normally stimulate any appreciable degree of mammary development in this species by contrast with, for example, the rat (20).

Teat-growth phenomena in the virgin female goat present certain points of great interest. In the first place, our observations indicate that the caprine ovary produces enough oestrogen to cause allometric teat growth quite soon after birth; in one case allometric teat growth was in progress only 41 days after birth. The data for all our females, during the allometric phase of teat development, agreed excellently with Huxley's simple allometric law (11). The abrupt cessation of teat growth in the majority of our animals at a time corresponding to the onset of the breeding season following their birth is of particular interest. In some animals teat growth ceased completely during the breeding season; in others a slight regression occurred (see Text-fig. 7). Allometric growth was resumed when the breeding season ended. The most probable explanation of this phenomenon is that the cessation of allometric

teat growth during the breeding season is due to some factor associated with the succession of oestrous cycles, and in this connexion the corpus luteum at once comes to mind. The duration of the oestrous cycle in the goat is approximately 21 days, and in such a cycle progesterone would be operative for a considerable proportion of the time. Cessation of allometric teat growth during the breeding season may therefore be due to two factors: (*a*) fluctuations in the degree of oestrogen stimulation, and (*b*) inhibition of teat growth by progesterone. This finding points to a distinction between the conditions which promote growth of the teat and mammary gland respectively, since the hormone secretion which results from a succession of regular oestrous cycles is such as to further mammary development in the goat (7).

Our results show that oestrogen causes the teats in young castrated and normal males to grow allometrically, but even after prolonged treatment they do not attain a length comparable with that reached in virgin females either normally or more rapidly under the influence of oestrogen (see Table 4). All our efforts to cause externally visible udder development in males failed even with very prolonged oestrogenic treatment and when progestational hormone was administered as well. In some cases, however, the treatment did stimulate the development of small amounts of alveolar tissue.

Table 4. *Maximum teat lengths of castrated male and normal virgin female goats, treated with diethylstilboestrol or its dipropionate*

Male goat	Approx. maximum mean teat length mm.	Female goat	Approx. maximum mean teat length mm.
Timothy*	18	Jill	37
Gargantua	20	Fanny	43
Punch	28	Jemima	44
Laurel	32	Poppy	45
William	35		

* Not castrated.

Our failure to cause udder growth in males is in agreement with the findings of de Fremery (4). The reason for the refractoriness of the male gland to oestrogen stimulation cannot be stated with certainty. Anatomical considerations may be involved however since according to Turner (21) no fatty pad representing the future udder is present in the male goat embryo (by contrast with the female) and the space into which the ducts may develop is therefore, according to him, limited. A qualitatively similar phenomenon was encountered in monkeys by Folley, Guthkelch & Zuckerman (10). They found that though oestrone administration would cause alveolar as well as duct development in female monkeys, only duct development was caused in most of the males studied.

By contrast with the male, we have found, in agreement with de Fremery (4), that treatment with oestrogen alone will, after a latent period which in our experiments has been somewhat variable, cause the development of an udder comparable in size with that formed in pregnancy. No progestational hormone was necessary.

Since complete growth of an udder which, as will be seen in Part II, is capable of normal function, can be caused in the female goat by administration of oestrogen without progesterone, it might appear that oestrogen alone will cause development of both ducts and alveolar tissue in the goat as in the guinea-pig. It must be remembered, however, that both de Fremery and ourselves used intact females, and even though de Fremery's experiments were carried out in the anoestrous season when no corpora lutea would be present in the ovaries, luteinization of the latter might have been stimulated by the oestrogen (22).

Our results on males, however, indicate that the goat is like the guinea-pig (1) and perhaps the monkey (23) (but see also Folley *et al.* (10)), in that development of both mammary ducts and alveolar elements is brought about by oestrogen.

The invariable formation of a capsule surrounding the diethylstilboestrol tablets in these experiments recalls the experience of Parkes & Young (15), who observed encapsulation of tablets of crystalline insulin, whether sterile or not, after implantation into rabbits. Curiously enough they found that tablets of amorphous insulin were completely absorbed with no encapsulation. They further state that tablets of oestrone or testosterone provoke no such local reaction even when left in the tissues for months. It is hard to see why tablets of diethylstilboestrol and crystalline insulin should undergo encapsulation, while tablets of oestrone, testosterone and amorphous insulin do not.

PART II. EXPERIMENTAL INDUCTION OF LACTATION *

De Fremery (4) was able to induce experimental lactation in virgin goats by injections of anterior pituitary extract containing prolactin (pigeon crop gland stimulating factor) following treatment with oestradiol monobenzoate which stimulated udder development. Lactation in virgin goats, particularly from high milking strains, is quite common, but de Fremery (4) states that in his animals lactation could never be induced by milking alone nor by oestrogen treatment without subsequent injections of anterior pituitary extract. Trautmann & Kirchhof (24) similarly induced lactation in virgin goats by oral administration of an extract of whole ovary followed by injections of prolactin. In the experiments described below, lactation invariably followed oestrogen treatment, no additional treatment with anterior pituitary extracts being necessary.

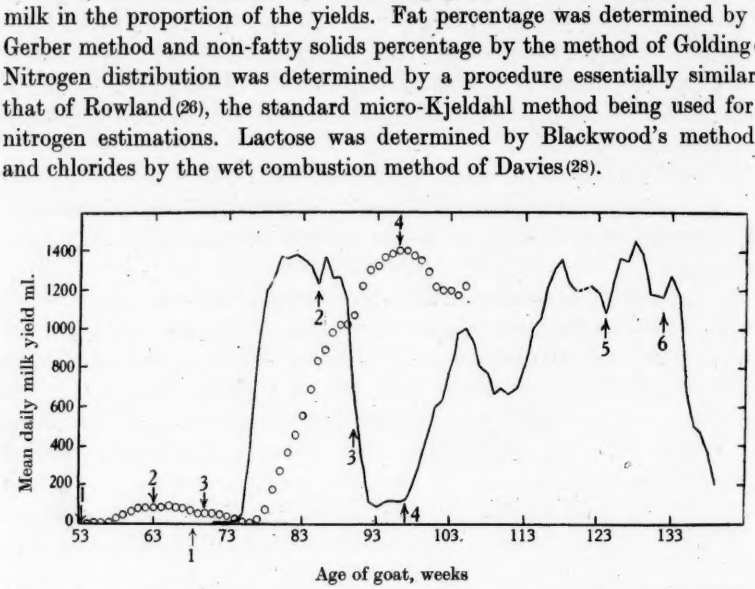
Experimental animals

Four virgin females were given oestrogen treatment and particulars are given in Table 3 (Part I). In addition, two controls were studied. The goats were milked twice daily at 8 a.m. and 3.30 p.m., and the yield measured in a measuring cylinder.

* Since the submission of this paper we have found that our results (8) are substantially confirmed by other authors, e.g. Lewis and Turner (*Proc. Amer. Soc. Anim. Prod.*, 1940, p. 63) and Walker and Stanley (*Anat. Rec.*, **78**, *Suppl.*, 142, 1940).

Milk analyses

At regular intervals throughout the experiment composite milk samples for analysis were obtained by combining portions of morning and evening milk in the proportion of the yields. Fat percentage was determined by the Gerber method and non-fatty solids percentage by the method of Golding (25). Nitrogen distribution was determined by a procedure essentially similar to that of Rowland (26), the standard micro-Kjeldahl method being used for all nitrogen estimations. Lactose was determined by Blackwood's method (27) and chlorides by the wet combustion method of Davies (28).



Text-fig. 9. The milk yields of the oestrogen-treated virgin goats Fanny and Jill. The unbroken line represents the lactation curve of Fanny. At ↑1 inunctions with 1·0 g. 1% diethylstilboestrol dipropionate ointment began three times weekly; at ↑2 this ointment was replaced by inactive base ointment; at ↑3 inunctions with 2·0 g. 1% diethylstilboestrol dipropionate ointment three times weekly began; at ↑4 the original dose of 1% ointment was applied. ↑5 denotes the last inunction and at ↑6 the goat was served. The circles trace the lactation curve of Jill. Daily milking began at 1↓ and inunction three times weekly with 1·0 g. inactive ointment base began at 2↓. Similar treatment with 1% diethylstilboestrol dipropionate ointment began at 3↓ and ended at 4↓.

Milk yield

In all four experimental goats, initiation of inunction was followed by copious lactation which set in after a latent period which varied somewhat from case to case.

The milk-yield curve for the non-pedigree goat Fanny is shown in Text-fig. 9. In this and in subsequent figures mean daily yields over weekly periods are plotted. A sudden increase in milk yield set in about 4 weeks after the first inunction. The yield rose rapidly to a level of almost 1400 ml. daily. The oestrogenic ointment was then replaced by inactive ointment base without

any great change in milk yield for about 3 weeks, after which an abrupt decline set in. When the daily yield had declined to about 600 ml. daily, oestrogenic ointment of double the original potency was applied, but the decline continued to a level of about 100 ml. daily, after which the curve flattened out. A return to the original 1% ointment was followed by an increase in milk yield to the previous maximum value, but at a much slower rate than when lactation was initiated. The yield still remained at a mean level of over 1200 ml. daily for some 9 weeks after inunction had ceased. The goat was served at this point and her milk yield thereafter rapidly declined.

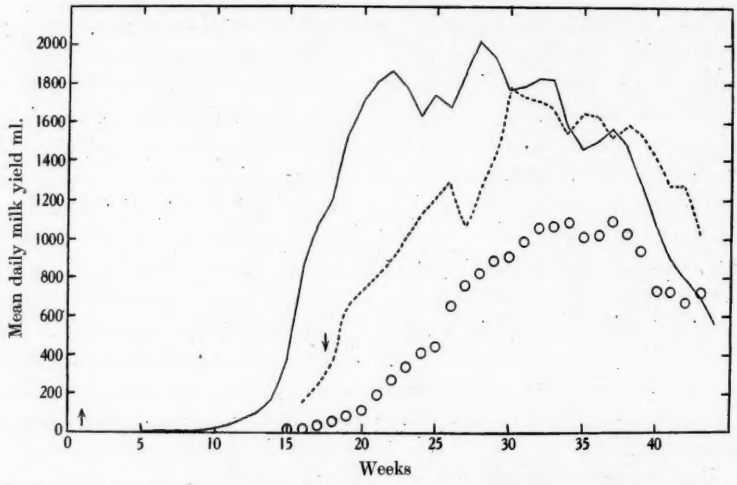
Text-fig. 9 also shows the milk yield curve for Jill, a pedigree British Alpine goatling. During a period of daily milking she never gave more than about 80 ml. per day, and this if anything decreased when inunction with base ointment started. This is regarded as of some significance, since many of her ancestors were high-yielding goats and she would therefore be more likely to yield milk without oestrogenic treatment than Fanny. Treatment with oestrogen was initiated when the goat was within a week of the same age as Fanny when the latter's treatment began, and after 7 weeks of treatment a rapid increase in milk yield occurred, though somewhat less rapidly than with Fanny. The milk yield rose to a maximum of 1400 ml. daily and then showed signs of a gradual decline. Unfortunately, this experiment came to an untimely end owing to the death of the goat from enterotoxaemia. It is important to note that in both these experiments lactation set in, increased rapidly and maintained a high level, while oestrogen was being regularly administered.

A well-controlled experiment was carried out with the twin pedigree British Saanen females Poppy and Pansy. Both were treated identically save that Poppy received oestrogenic ointment and Pansy inactive ointment base. The milk-yield curves are shown in Text-fig. 10. It will be seen that milk secretion began much earlier after the beginning of the experiment in the oestrogen-treated goat than in the control, actually after 8 weeks as compared with 28 weeks. The yield of the experimental goat which hitherto had remained low began to rise rapidly at about 25 weeks after inunction began and quickly reached a maximum of nearly 2000 ml. daily, at which level it remained for about 28 weeks. The yield of the control goat increased much more slowly and never exceeded more than about 1000 ml. daily, about one-half of the maximum attained by her twin.

The milk-yield curve for Snowwhite, an untreated, pedigree British Saanen goatling which was 67 days older than the twins, is also for comparison shown in Text-fig. 10. Her yields are given for the same dates as the twins. The udder of this virgin increased in size and produced milk soon after the experiment with the twins began, and it was possible to plot her yields along the same time axis. Daily milking began at the point at which her lactation curve starts, though she had been observed to have been in milk for some

time previous to this. Her milk yield rose almost but not quite to the same maximum as the oestrogen-treated twin but at a noticeably lower rate.

In the case of the fourth experimental goat, Jemima, a non-pedigree female, inunction was continued for a considerable time, a matter of 4 months, before she began to secrete milk. Her milk yield rose much more slowly than any of the others, reached a maximum of only 600 ml. daily, and a gradual decline set in almost at once. It may be mentioned that in this goat an increase in teat growth did not set in for some months after the commencement of inunction.



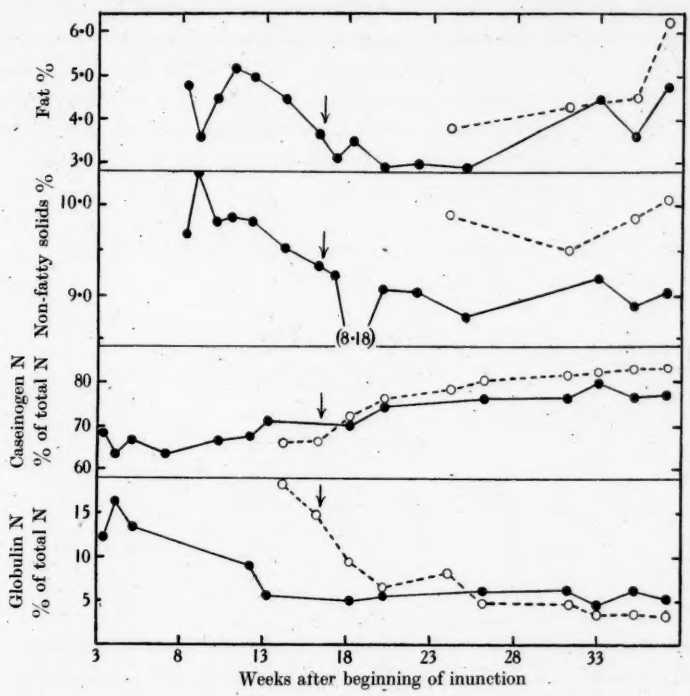
Text-fig. 10. The milk yields of the oestrogen-treated virgin goat Poppy, her twin control Pansy, and the untreated virgin goat Snowwhite. The unbroken line represents the lactation curve of Poppy, which received three inunctions weekly of 1·0 g. 1% diethylstilboestrol ointment beginning at ↑ and ending at ↓. The circles trace the lactation curve of Pansy which received corresponding inunctions with inactive ointment base, while the dotted line represents the lactation curve of Snowwhite, an untreated virgin goat 67 days older than the twins.

Milk composition

The values for fat and non-fatty solids percentages, and of caseinogen and globulin nitrogen expressed as percentages of the total nitrogen, in the milk of the twins Poppy and Pansy are plotted in Text-fig. 11. Some explanation of the significance of the latter two quantities (which are hereinafter called the casein and globulin numbers) may be called for at this point.

In normal cows' milk the casein and globulin numbers remain relatively constant. The former usually lies between 76 and 80, while the latter is rarely greater than 5 (29, 30). In abnormal milk, such as that secreted by glands infected with mastitis, the casein number is lower and the globulin number higher than normal (30). From what is known of the composition of bovine secretions of the colostral type (31, 32), it is clear that such secretions would

be characterized by lower casein and higher globulin numbers than normal milk. The transition from colostrum to milk must therefore be accompanied by an increase in the casein number and a reduction in the globulin number. From the data of Bergman & Turner (33) on the composition of goat's colostrum it may be concluded that the above considerations also apply to the goat, though from the figures given by Knowles & Watkin (34) (see Table 5), it appears that the average casein number for normal goats' milk (74·2) is slightly lower than for normal cows' milk. Determinations of casein and



Text-fig. 11. Composition of the milk of the twin virgin goats Poppy and Pansy. The black points relate to the experimental goat Poppy which received three inunctions weekly with 1·0 g. 1% diethylstilboestrol ointment. The circles relate to the control goat Pansy which received corresponding treatment with inactive ointment base. ↓ denotes the last inunction.

globulin numbers therefore enable one to follow the transition from secretions of a colostral type to true milk, and are of value in distinguishing between the two types of secretion.

Text-fig. 11 shows that the milk of the oestrogen-treated goat was, during the first 14 weeks of inunction when the milk yield was low, characterized by a low casein number as compared with normal goats' milk, and the globulin number high as compared with the normal for cows' milk. (We know of no values for the globulin number for normal goats' milk, obtained by a method similar to ours and therefore comparable. It is safe to assume, however, that

goats' milk will not differ much from cows' in this respect.) These quantities gradually attained constant and presumably normal values, however, and remained normal for the rest of the artificial lactation period. Fat and non-fatty solids percentages were high at the outset and declined somewhat during the first half of the lactation period.

These changes are qualitatively similar to those which occur normally during the transition from colostrum to milk in the goat (33). After parturition, however, the transition takes only a few days, while the alterations in milk composition observed in this experiment were more gradual.

Similar changes in casein and globulin numbers occurred in the milk of the control, but normal values were attained quicker. The milk of the control goat was, during the latter half of the experiment, slightly superior in quality to that of the oestrogen-treated animal, but the milk of both animals compared favourably in composition with the average for normal goats' milk given in the last column of Table 5.

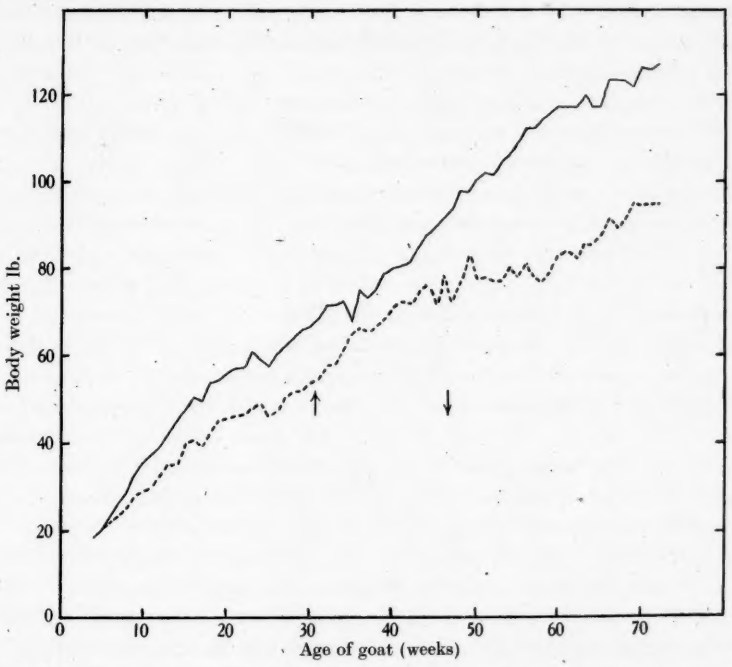
Table 5. *Composition of the milk of the virgin goat Fanny*

	Inunction thrice weekly with 1·0 g. of 1% ointment, 1st period	Inunction with ointment base		Inunction thrice weekly with 1·0 g. of 2% ointment		Inunction thrice weekly with 1·0 g. of 1% ointment, 2nd period		No treatment		Average composition of goat's milk under English conditions (Knowles & Watkin (34))
	4. x. 39	23. xi. 39	5. xii. 39	16. i. 40	23. i. 40	6. ii. 40	13. ii. 40	3. ix. 40	1. x. 40	
Fat %	—	5·90	6·85	6·30	7·05	—	5·55	4·03	3·70	4·50
Non-fatty solids %	—	10·29	10·92	10·93	11·44	—	10·85	8·79	8·99	9·02
Lactose %	5·48	5·46	—	—	—	5·46	5·50	—	—	4·08
Chlorides %	0·093	0·099	0·097	0·103	0·094	0·099	—	—	—	0·105
Total N, mg. %	597	699	780	874	919	822	780	575	636	522
Caseinogen N, % of total N	79·9	76·9	78·0	76·3	74·7	73·8	76·0	82·8	75·6	74·2
Globulin N, % of total N	—	4·45	5·00	6·18	7·30	6·69	—	5·15	6·43	—

The composition of the milk of Fanny at different times during her experimental period is given in Table 5. For comparison the last column shows the average composition of goat's milk under English conditions (34). In general, the composition of the milk of this goat was superior to the figures given in the final column in that the percentages of fat, non-fatty solids, and lactose and casein number were higher and the chlorides lower than the latter. In explanation of the last point it should be stated that the lactose and chloride content of milk vary inversely (35). Towards the end of the period of inunction with 2% ointment the figures suggest a slight lowering of casein number and a corresponding rise in globulin number, which would suggest that treatment with too much oestrogen tends to promote a colostral type of secretion in the goat. This is in agreement with the contention of de Fremery (4).

The question of experimental induction of lactation in the male can be disposed of briefly. In only two (William and Laurel) of the males which received oestrogen treatment (Part I) was any mammary secretion produced

in response to daily milking, and, as would be anticipated from the lack of udder development, the yields were minute. Laurel never produced more than a drop or two of a fluid having the superficial appearance of milk, though from William amounts of the order of 2 ml. daily were obtained at times, thus permitting a few analyses. Figures obtained at various times for the secretion were as follows: % total solids 14·56, 8·63, 13·37; % fat 4·50, 3·87; mg. total nitrogen per 100 ml. 581; casein number 78. These figures indicate that the mammary secretion produced by William was to all intents and purposes normal milk.



Text-fig. 12. Growth curves of twin virgin goats Poppy and Pansy. The broken curve refers to the experimental goat Poppy and the unbroken curve to the control goat Pansy. ↑ denotes the beginning of inunction three times weekly with 1·0 g. of ointment containing 1% diethylstilboestrol in the case of Poppy and 1·0 g. inactive ointment base in the case of Pansy. ↓ denotes the last inunction.

Oestrogen treatment and body growth

Zondek (36) has shown that oestrogen treatment depresses the growth rate of young rats, and Noble (37) found that this is true of the synthetic oestrogen diethylstilboestrol. The possibility that treatment with diethylstilboestrol such as we have described would inhibit the growth of immature goats is therefore of some practical importance. The only reliable data on this question we possess at present were obtained in the experiment on the twins. The growth curves for these goats are shown in Text-fig. 12. The goat selected for oestrogen

treatment had been from birth somewhat lighter than her sister, but the curves give no reason to conclude that the disparity became more marked during the inunction period. The growth rate of the oestrogen-treated animal, it is true, declined noticeably after the inunction had ceased, but this coincided with a period when she was secreting considerable quantities of milk, and the decline in growth rate was probably due to the strain on her metabolism associated with lactation rather than to the direct action of the diethylstilboestrol.

Discussion

These experiments show that in the virgin goat external application of suitable doses of oestrogen to the skin surrounding the base of the teats results, after a latent period during which growth of the udder and teat occurs, in the induction of prolonged artificial lactation periods during which considerable amounts of normal milk are produced. In connexion with these experiments two important points should be noted. First, under our conditions, lactation was induced without treatment with an anterior pituitary extract. In this respect our findings differ from those of de Fremery (4) who states: 'If, however, the preliminary treatment with oestrogenic hormone is not followed by a short but intensive course of injections with prolactin, only a serous fluid can be expressed from the udders.' Secondly, lactation was initiated, increased in intensity and remained at a high level while oestrogen administration actually continued. This appears to be quite at variance with the classical view according to which oestrogens exert an inhibitory action on lactation. It has, however, been shown in experiments on lactating cows (38, 39, 40) that lower levels of oestrogen than are necessary for terminating lactation exert not an inhibitory, but, on the contrary, a galactopoietic effect which principally manifests itself by an increase in the solids content of the milk. This effect is probably exerted through the anterior pituitary lobe. Our present results therefore become explicable in the light of this enlarged conception of the role of oestrogens in lactation. The oestrogen in the present experiments has undoubtedly (*a*) promoted growth of the udder, and (*b*) stimulated the production by the anterior pituitary of the lactogenic or galactopoietic (41) hormones necessary for the initiation and maintenance of lactation. It is possible that de Fremery's failure to initiate experimental lactation in the absence of treatment with anterior lobe extracts was due to the use of doses of oestrogen so high as to be incompatible with lactation.

The changes in the composition of the milk secreted during artificial lactation are of great interest. In the early part of the lactation period changes in composition occur which qualitatively resemble those occurring in a natural lactation during the colostral period. However, in the oestrogen-induced lactation these changes occur much more slowly, and none of our samples had the huge protein content of early colostrum. The milk secreted during the greater part of the artificial lactation periods was of excellent chemical quality, comparing, as regards the three major milk constituents, fat, lactose

and casein, very favourably with the average figures for the composition of the milk of pedigree English goats.

SUMMARY

1. Observations on five animals indicate that the teats of immature castrated male goats grow isometrically. Less extensive data on three animals allow of the tentative conclusion that the same is true of immature normal males. In the male goat therefore teat growth appears to be uninfluenced by the testes.

2. Administration of diethylstilboestrol or its dipropionate to normal or castrated immature male goats causes the teats to grow allometrically for a time.

3. No externally visible udder growth occurred even when oestrogen treatment was prolonged for periods of over a year and was supplemented by progesterone or ethinyltestosterone. Whole mounts of glands from treated animals indicated that some mammary growth had occurred. In two cases microscopic examination showed the presence of alveoli. No explanation can be offered of the failure to develop the udder in the male goat experimentally.

4. Endocrine activity of the ovary as evidenced by a change from isometric to allometric teat growth often manifests itself in the young female goat at an early age. In one case allometric teat growth was in progress at 41 days of age. During the allometric phase the data agree with the simple allometric law.

5. During the rutting season following its birth, teat growth ceases completely in the female goat; allometric growth is resumed when the rutting season ends. It therefore appears that the corpus luteum inhibits teat growth.

6. Administration of diethylstilboestrol or its dipropionate (by inunction of the udder region) causes, in the virgin female, an increase in the rate of teat growth accompanied by udder growth.

7. In virgin females the above treatment was followed, after a latent period during which udder growth occurred, by prolonged lactation, at the height of which the daily milk yield was of the order of 1·5–2 l. No treatment with anterior pituitary extract was necessary. In normal or castrated males the treatment resulted in the secretion of only minute quantities of milk.

8. Lactation was initiated and increased in intensity while oestrogen administration continued. It is thought that low doses of oestrogen exert a galactopoietic effect through the anterior pituitary.

9. During the first part of the artificial lactation period, the changes in milk composition were similar to those characteristic of the colostral period. Subsequently, milk of excellent chemical quality was secreted.

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CORRIGENDA

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THE PLATE COUNT AND METHYLENE-BLUE REDUCTION TEST APPLIED TO MILK

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Arising from the discovery of an error in calculation the following are published in the interests of accuracy. It is emphasized that *the findings are in no way affected.*

P. 299. L. 15. Equation (1) should read:

$$X = 5{\cdot}816 - 0{\cdot}0039y$$

L. 17. Equation (2) should read:

$$Y = 983{\cdot}29 - 134{\cdot}72x$$

L. 21.	*For* 0·114	*read* 0·117
L. 22.	*For* 1·3	*read* 1·309
L. 24.	*For* 114 min.	*read* 134·72 min.
L. 25.	*For* (1 hr. 54 min.)	*read* (2 hr. 15 min.)
L. 34.	*For* 346,000	*read* 382,000
L. 35.	*For* 22,400,000	*read* 7,136,000

P. 300. Table 21 should read as follows:

$X = 5{\cdot}816 - 0{\cdot}0039y$			$Y = 983{\cdot}29 - 134{\cdot}72x$		
When R.T. is in hr.	Mean $\log_{10}$ plate count will be	Approx. arithmetic equivalent	When $\log_{10}$ plate count is	Approx. arithmetic equivalent	Mean R.T. will be in hr.
9	3·71	5,100	3·29	1,900	9
8	3·94	8,800	3·74	5,400	8
7	4·18	15,000	4·18	15,000	7
6	4·41	26,000	4·63	42,000	6
5½	4·53	34,000	4·85	71,000	5½
5	4·65	44,000	5·07	118,000	5
4½	4·76	58,000	5·29	197,000	4½
4	4·88	76,000	5·52	329,000	4
3	5·11	130,000	5·96	918,000	3
2	5·35	223,000	6·41	2,559,000	2
1	5·58	382,000	6·85	7,136,000	1
	$x = \log_{10}$ plate count		y = reduction time in minutes		

P. 301.

L. 2.	*For* 5·54	*read* 5·582
L. 3.	*For* 7·35	*read* 6·8534
L. 13.	*For* 78 min.	*read* 76 min.
L. 14.	*For* 34 min.	*read* 40 min.
L. 15.	*For* 78 and 34	*read* 76 and 40
L. 17.	*For* 78, 34 and 56	*read* 76, 40 and 58
L. 23.	*For* 0·1611	*read* 0·1654
L. 24.	*For* 0·3719	*read* 0·3147
L. 25.	*For* 0·2665	*read* 0·2401

Corrigenda

P. 301. L. 26. *For* 0·3719 and 0·1611 *read* 0·3147 and 0·1654
L. 29. *For* 0·1856 and 0·4285 *read* 0·1905 and 0·3626
L. 30. *For* 'less' *read* 'greater'
For (0·3071) *read* (0·2766)
L. 31. *For* 'to the plate count over R.T. 30' *read* 'to R.T. 30 over plate count'

P. 302. Table 22.

The regression equations should read:

$$X = 5{\cdot}816 - 0{\cdot}0039y, \quad Y = 983{\cdot}29 - 134{\cdot}72x$$

Plate count should read:

Col. 2	Col. 4
76	40
54	28
44	23
34	18

R.T. 5 should read:

Col. 1	Col. 3
0·1654	0·3147
0·1169	0·2225
0·0955	0·1818
0·0740	0·1409

R.T. 30 should read:

Col. 1	Col. 3
0·1905	0·3626
0·1347	0·2563
0·1099	0·2092
0·0853	0·1623

Table 23. Right-hand column should read:

58
41
33·5
26

Table 24. Under '5 min.' (middle column) should read:

0·2401 log
0·1697
0·1387
0·1075

Under '30 min.' (right-hand column) should read:

0·2766 log
0·1955
0·1596
0·1238

637.127.6:576.809.3

286. THE PLATE COUNT AND METHYLENE-BLUE REDUCTION TEST APPLIED TO MILK

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(With 1 Figure)

SYNOPSIS

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Addendum I. Normality of distribution of replicates
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General Summary
Summary of Addenda
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Introduction

Until 1 January 1937 milk was graded in England and Wales by means of the plate count. For many years bacteriologists have been aware of the errors and other drawbacks of this method in routine work, which should have precluded its use in classifying milks into any but broad grades. Many were acutely uneasy because of the precision which some workers, both lay and scientific, attributed to the individual results of a test which apparently had a high variability and, in consequence, poor reproducibility. Low variability (good reproducibility) in replicates of the same sample is, of course, the first essential of any method used in comparing one sample with another. Whatever else may be claimed for the methylene-blue test, it certainly has the virtue of simplicity of technique, a quality of great importance in a test intended for routine use on a large scale.

It has been demonstrated that, with plate counts reported in the usual manner, the errors between workers and between laboratories and the error of a single worker in examining portions of the same milk, are large(1). In spite of this, the results of the test continued to be misused. Thus, for example, milk 'A' with a plate count of, say, 70,000 per 1 ml., was judged to be superior to milk 'B' with a plate count of 105,000 per 1 ml. Actually it has been shown that on the basis of a single examination, there is statistically no significant difference between the two, for unless the difference in the plate counts exceeds a ratio of 1 : 1·5 we are not justified in asserting the superiority of the one sample over the other(1). It was also established(1) that under uncontrolled conditions of transit, the results, from different laboratories, of tests on samples of the same milk cannot justly be compared.

It must, nevertheless, be said that the plate count could not be wholly condemned, for, despite its limitations as a basis for official grading and for the apportionment of cash payments, it is valuable for advisory purposes.

In January 1937 the Ministry of Health in England abandoned the use of the plate count (except for pasteurized milk) and substituted a modified form of the methylene-blue reduction test. The chief modifications consisted in the introduction of half-hourly inversions of the closed tubes until decolorization commenced and in requiring exposure of the milk samples to atmospheric shade temperature for a stated time before testing(2).

The fact that, in spite of its apparent limitations, the quality of much of our milk supply had been vastly improved by the use of the plate count over a period of years, and that its language was generally understood by producers of milk and others interested, made it desirable that an exploration of the subject should be made under field conditions, in as many parts of the country as possible.[1] It was obviously desirable to know whether, in the field, the standards set by the new methylene-blue test approximated to those of the old plate count. Whichever test is used, judgement ought, of course, to be

[1] For various reasons the publication of this paper in full has been delayed.

based on a series of samples and not on the result of a single test, a desideratum which in practice is not always observed. It was, moreover, not generally appreciated that the two tests measure different properties of the milk flora.

A major claim for the modified methylene-blue test was its reproducibility, a property of first importance. It was clearly desirable to test this assertion on a large scale, as, although it had been shown that the variability due to observers in the same laboratory was insignificant, there was no evidence of its magnitude between different laboratories. The overpowering effect of temperature upon bacterial growth in milk, which may obscure all other influences, was well known, but the old methods of management of milk samples paid little attention to this important factor. It was, therefore, further desirable to test the value of the methylene-blue test as an index of the conditions of production, when the temperature factor was, as far as possible, eliminated.

Finally, there appeared to be little evidence on which to assess the comparative capacities of the plate count and the methylene-blue reduction test to differentiate samples of milk of various levels of plate count or reduction time.

With the above objects in view the following co-operative experiments were designed in March 1936. All the participants, who were stationed in widely separated districts in England and Wales (i.e. north, south, south-east, south-west, east, west, Midlands, Wales and London District) undertook to follow categorically for each experiment the agreed instructions which were sent to them, and to record any variations in the methods of technique which arose unavoidably.

The media were all made to common formulae, the temperatures of incubation were uniform, the methods and technique for counting were also uniform and the transit conditions of samples were stipulated. The experiments fell into two series extending over the summer of 1936 and the winter of 1936–7, and some further results were obtained during the winter of 1937–8. The experiments also afforded an opportunity to investigate two further points, namely, the comparative variation of replicates using plain agar and 1% milk agar, and the comparative accuracy of different methods of assessing the final reported plate count.

The following techniques were used:

Methylene-blue test, 37° C.	Wilson (2)
Plate count	Ministry of Agriculture (3)
Keeping quality test	Ministry of Agriculture (3)
Methylene-blue test (15·5° C.)	Raise tubes to 60° F., incubate in air and invert every half-hour 10.0 a.m.–6.0 p.m.
Standard (plain) agar	Ministry of Health (4)
Milk agar	Ministry of Agriculture (3)

In the technique used (3) plates were incubated for 72 hr. at 37° C. Hiscox, Hoy, Lomax & Mattick (7) showed that with pasteurized milk the count was

49% higher after 66 hr. at 37° C. than it was after 48 hr. incubation. With raw milk the increase was less. They also claimed that visibility of the colonies was much improved by the extra incubation. Wilson (2) obtained similar results, but the increase was not so great and he found no difference in the accuracy of counting at the two stages. Stability appears to be reached if 72 hr. at 37° C. incubation is followed by 48 hr. at 22° C. (7). Barkworth (29) obtained significantly higher counts by incubating for 72 hr. at 22° C. after 72 hr. at 37° C. It is also known that milk which has been cold stored may contain large numbers of organisms whose growth may be poor even at 22° C. The Ministry of Health (27) now prescribe 48 hr. incubation at 37° C.

The instructions also gave detailed and precise directions on the age of the samples at each test, the method of storage up till time of testing, the tests to be applied, and the method of recording.

A schedule of tests in the three main sections of the experiment is given in Table 1.

Table 1. *Schedule of tests*

	Exp. I. *Any* milk			Exp. II. Special samples		Exp. III. Inspected samples	
Time of milking	a.m. (received same day)	a.m. (received next day)	p.m.	a.m. (in ice-box)	p.m.	a.m.	p.m.
Transit	Air temp. till 5 p.m.* Test Cold store overnight Re-test	—	Air temp. till 10 a.m.*	Record arrival temp. Fat test	Record arrival temp. Fat test	2 hr. transit. 60° F. till 6 p.m.	2 hr. transit. 60° F. overnight
Standard agar	In duplicate at 5 p.m.	In duplicate at 10 a.m.	In duplicate at 10 a.m.	—	—	—	—
Milk agar	In duplicate at 5 p.m.	In duplicate at 10 a.m.	In duplicate at 10 a.m.	In duplicate at 10 a.m.	In duplicate at 10 a.m.	In duplicate at 10 a.m.	In duplicate at 10 a.m.
Reductase:							
37° C.	10 a.m.	10 a.m.	10 a.m.	In duplicate, at 10 a.m.	In duplicate at 10 a.m.	10 a.m.	10 a.m.
15·5° C.	5 p.m. and 10 a.m.	10 a.m.	10 a.m.	In duplicate at 10 a.m.	In duplicate at 10 a.m.	10 a.m.	10 a.m.
Keeping quality started at	5 p.m.	10 a.m.	10 a.m.	—	—	10 a.m.	10 a.m.

All reductase tests to be observed for 7½ hr. and recorded as: (P.), (V.P.), (V.V.P.) or (R.).
Record temperatures of 60° F. incubator.
At 60° F. invert methylene-blue tubes half-hourly from 10 a.m. to 6 p.m.

* Samples travelled to the laboratory by post, rail and road in sample bottles.

Statistical treatment

The chief functions of statistical analysis in experiments such as these are to provide a sound basis for experimental design, to ensure that the desired information can be obtained and that any interesting effects can be tested for significance. Further, there must be adequate methods for the reduction of the data, and for testing the significance and estimating the magnitude of the effects indicated by the results. It is these two latter points with which we are more particularly concerned here. While there are certain statistical rules which must be followed, a given method is not necessarily universally applicable. Certain data will follow one type of distribution and others

another, necessitating either the application of a different method of treatment or the transformation of the variate into some other unit of measurement which will enable us to use the old method. One of the main objects of the experiment was to assess the variability of the different tests. In connexion with milk tests variability is usually taken to mean some measure of the difference between replicate determinations on the same sample; the simplest and commonest method of assessing variability is to calculate the *standard deviation* (S.D.).

If the most useful available statistical methods are to be valid two conditions should be satisfied. The data should, at least approximately, be normally distributed, and the S.D. should be independent of the mean. The former condition is necessary in almost all significance tests on small samples and is useful even with large samples, because normal frequency tables can then be used in conjunction with the S.D. The latter condition is necessary in applying *analysis of variance* methods.

Further, we were interested in the value of the average plate count corresponding to a given reduction time and vice versa, i.e. in regression. The calculations will be greatly simplified if the regression is linear, so that a given average increase in plate count corresponds to a given increase in reduction time at any level of reduction time and vice versa.

These three conditions are, in fact, satisfied by the measure we have chosen, as is shown in Table 17.

Keeping quality at 60° F.

This test does not appear in Table 17, but it has been proved (5, 6) that there is a linear relation between the logarithm of the plate count and the keeping quality in hours or days. From arguments below it would follow that there is also a linear relation between the reduction time at 37° C. and the keeping quality at 60° F.

Methylene-blue test

The results are recorded as time taken to reduce the dye from blue to white (reduction time, R.T.). In Table 17 data have been calculated for the actual values of R.T. when observed at 5 min. intervals, and for the logarithm of R.T. A comparison might suggest the possible use of $\sqrt{\text{R.T.}}$, but any advantage so gained would be slight, and it is preferable to use the actual values whenever possible. This has been done.

Plate count

Robertson & Frayer (6) used the logarithms of the plate counts, a practice which has since been followed by many workers. Mattick, McClemont & Irwin (1) used the arithmetical values, the square root and the logarithm of the colony numbers and concluded that the use of the arithmetic values was unsound. There was little to choose between the other two units. Wilson (2) used the actual values of the colony numbers. In the present work we have used the logarithms of the reported counts.

Several reasons may be advanced for using the logarithms. First, since bacteria increase by dividing into two, it is not the actual increase but the 'number of times' of increase which is of general interest. Thus:

Arithmetic value	(*a*)	5,000	increases to	10,000.	Net increase	5,000
Log values	(*b*)	3·6990	,,	4·0000	,,	0·3010
Arithmetic value	(*c*)	100,000	,,	105,000	,,	5,000
Arithmetic value	(*d*)	100,000	,,	200,000	,,	100,000
Log value	(*e*)	5·000	,,	5·3010	,,	0·3010

When (*a*) and (*c*) are compared arithmetically the increase of 5000 is the same in both cases, but the relative change is much greater in (*a*). The logarithmic values seem to give a more reasonable picture of what actually happens, for they increase by a constant amount when the arithmetic values increase in a constant ratio, at any level whatever.

Secondly, there is the question of the S.D. Table 17 shows that arithmetical values are unsuitable for this purpose, as the correlation between S.D. and mean is here highly significant, 0·6338.[1]

Thirdly, it will be shown that there is a linear relation (see p. 299) between R.T. and the logarithm of the count but not between R.T. and the actual count.

Lastly, there is the question of the form of distribution of the data. It has been accepted that replicate counts or a series of plate counts of samples having nearly the same bacterial content may be treated by using the actual values, and we shall presently offer statistical evidence that the distribution of the replicates is such that they can be regarded as normal either on an arithmetic or on a logarithmic basis. It is not, of course, possible for both the actual and the logarithmic values to be exactly normally distributed, but both could approach normality with sufficient approximation, and this is what occurs.

The distribution of the values of the replicates is a 'within sample' variation; a different situation arises when we consider variation between samples, e.g. the result of a series of samples of all levels of count. Robertson & Frayer (6) have shown that if the plate counts of a large number of samples are plotted logarithmically the distribution curve is normal, and counts deviating from the mean by two or three times the standard deviation will always have positive and reasonable values. If the actual values are plotted the resulting curve is not normal, and the application of the normal table to the standard deviation will result in impossible negative values.

There is, therefore, statistically no evidence to warrant the use of arithmetic values with the plate count. On the other hand, there is much evidence, both theoretical and practical, to indicate that the logarithm is the proper unit, and we have used it.

[1] Ref. (1) gives a correlation of 0·7 between S.D. and mean.

EXPERIMENT I. GENERAL AGREEMENT OF PLATE COUNT AND METHYLENE-BLUE TESTS

Object

The primary object of this experiment was to find whether the standards set by the modified methylene-blue reduction test (5½ hr. winter, and 4½ hr. summer) approximated to those set by the plate-count method, which was used under the old Milk (Special Designations) Order, 1923. For this purpose milks from graded farms under control as well as from quite uncontrolled sources were used, so long as they conformed to the protocols of the experiment. Reference to Table 1 will show that in addition to the principal tests (plate count and methylene-blue test at 37° C.), other tests such as the keeping quality test and the methylene-blue test at 15·5° C. were used. In some cases the tests were made in duplicate and milk agar[1] was used as well as the standard (plain) agar of the Ministry of Health. In seeking to compare the plate count and the methylene-blue test we have no absolute standard of reference, and it was thought that the subsidiary tests might provide additional information which would assist in overcoming this difficulty and provide a form of counter-check.

Statistical treatment of data

The treatment of data for both plate count and reduction time presented difficulties owing to the presence of 'indefinite' observations, the limits of actual observation for routine plate counts being 500,000 per 1 ml. and (in most cases) 7½ hr. for reduction time. The difficulties become obvious when it is realized that although the results of many samples were within the limits of definite observation for both tests ('definite' samples), there was also a number of samples at all levels of plate count which had indefinite reduction times and vice versa. The relation between plate count and reduction time is obviously dependent not only on the definite observations but also on the true value of the 'indefinite' observations. The average reduction time for samples of a given plate count may be represented by the mean of the known reduction times, but is more truly the mean reduction time of all samples including the indefinite values. A completely accurate statement of the overall position cannot be calculated because of these indefinite observations.

On this basis the relation could only be seriously disturbed (*a*) if a high

[1] At the time that these experiments were planned and commenced (1936) standard (plain) agar was the official medium as prescribed by the Ministry of Health (4), but milk agar (3) had been the standard medium for plate counts since 1932. The superiority of milk agar had been definitely proved (7, 8), and when the revised edition of Memo. 139/Foods was issued in January 1937 it was found that a yeastrel-peptone-1% whole-milk agar had been adopted.

It is unlikely that there will be any essential difference in the results obtained on these two milk agars, and our figures may therefore be accepted as applicable to samples taken under the Milk (Special Designations) Order, 1936.

proportion of samples with reduction times between 4 and 7½ hr. had indefinite counts, or (*b*) if many samples of high plate count had indefinite reduction times. In an attempt to give proper weight to indefinite observations, an arbitrary figure of 6·75 was allotted for the logarithm of all indefinite plate counts, *c.* 3½ millions, and 9 hr. for indefinite reduction times. The latter figure is based on the assumption that most of the indefinite observations would have fallen between 7½ and 12 hr. Proceeding on this basis the observations can be referred to as 'definite' or 'indefinite', the term 'definite' implying that all samples had definite observed plate counts and also definite reduction times. In the indefinite class, either count or reduction time or both were beyond the limits of precise observation. This difficulty does not arise with keeping quality tests because there is no absolute limit to observation. These tests can be continued for 4 or 5 days or longer if necessary.

Results for the methylene-blue test at 15·5° C. require explanation. The tubes were observed half-hourly between 9 a.m. and 6 p.m., but there were no observations between 6 p.m. and 9 a.m. Many samples decolorized during the long interval overnight, and the results in such cases are indefinite observations. All such indefinite samples have been excluded in dealing with reduction time at 15·5° C. The effect of this will be discussed when dealing with correlations.

The data have been examined by two methods. (*a*) By noting the agreement or disagreement of the old and new methods of grading, (i) taking the upper plate count limit for Grade A milk, 200,000 colonies per 1 ml., and (ii) (the new methylene-blue test) taking the lower reduction time limit to be 4½ hr. during May–October and 5½ hr. in the winter months. (*b*) By correlating various pairs of tests.

Table 2. *Comparison of grading*

	Summer	%	Winter	%
(*a*) Passed or failed both tests	(1003)	87·3	(631)	84·6
(*b*) Passed plate count, failed R.T.	(105)	9·1	(87)	11·7
(*c*) Passed R.T., failed plate count	(41)	3·6	(28)	3·8
	(1149)		(746)	

Figures in brackets are numbers of samples.

Comparison of grading

Wilson (2) indicates that the reduction time of milks with plate counts not exceeding 200,000 per 1 ml. should exceed 5½ hr. in summer and 6½ hr. in winter. Memo. 139/Foods (27) states that milk must not decolorize methylene blue within 4½ hr. in summer and 5½ hr. in winter. Our figures show that the latter values correspond closely to a limit value for the plate count of 200,000 per 1 ml. (Table 2). The closeness of this agreement is the more remarkable when it is recalled that the plate count estimates numbers of bacteria irrespective of their action on milk, while the reduction time is influenced by

types as well as numbers and is also affected by their activities previous to testing.

It is important to note that previous to the plate test, samples had had a minimum of 12 hr. exposure to atmospheric (shade) temperature, whereas previous to 1937 all *official* samples were packed in ice. The technique of the Ministry of Agriculture(3) has always required 18–28 hr. at atmospheric temperature.

Correlations

Correlations have been calculated for each of the twelve contributing laboratories, for winter and summer results. Some of the individual series contained only a few samples, and only the summarized results are shown in Table 3. In each case a.m. and p.m. samples were treated separately and further subdivided according to the storage conditions and the time of testing. The plate counts used were those from milk agar(3); where samples had been tested in duplicate, the mean logarithm was used.

Our figures are not strictly comparable with those given by Wilson(2) because he used composite samples (mixed a.m. and p.m. milk) and his correlations are not subdivided according to transit conditions and age of testing. The methods and criteria for the keeping quality test were also different.

It must be remembered that the correlation coefficient measures only the degree of agreement between two variates, and by itself does not prove that either variate is a correct measure of the item we wish to assess.

(i) *Plate count and reduction time at* 37° C.

As might be expected from Table 2 the general level of correlation is good, except the values for p.m. samples in summer, a high proportion of which came from one laboratory where it appeared that difficulties had arisen with the agar medium.

(ii) *Plate count and keeping quality*

The keeping quality test is subjective, but despite this, good agreement has been obtained, and the figures compare well with those obtained by Barkworth(5), who examined 5000 a.m. and 5000 p.m. samples and recorded correlation coefficients of 0·54 and 0·50 for a.m. and p.m. samples respectively, the plate counts, in this case, being on plain agar.

(iii) *Keeping quality and reduction time at* 37° C.

It appears that there is little difference in the relation of plate count to keeping quality and that of reduction time to keeping quality. Both these relations have values of the same order as that of plate count and reduction time at 37° C.

(iv) *Reduction time at* 37° C. *and reduction time at* 15·5° C.

It is obvious that the different temperatures of incubation will favour different types of bacteria and therefore the correlation may not be good.

Table 3. *Correlation coefficients between various pairs of tests*

Correlation	Class	Summer				Winter			
		No. of samples	Including indefinite	No. of samples	Definite	No. of samples	Including indefinite	No. of samples	Definite
Plate count (log) and R.T. 37° C.	(*a*) a.m. received same day (stored overnight in refrigerator)	536	−0·7927	310	−0·7393	255	−0·7559	288	−0·5643
	(*b*) a.m. received next day (overnight at air temperature)	258	−0·7892	325	−0·7429	54	−0·8438	153	−0·6627
	(*c*) p.m. (overnight at air temperature)	208	−0·4919	82	−0·4089	263	−0·5191	40	−0·6450
Plate count (log) and keeping quality	(*a*) As above	340	−0·7888	239	−0·7281	105	−0·6104	97	−0·5362
	(*b*) ,,	387	−0·7328	345	−0·6709	154	−0·6482	148	−0·5962
	(*c*) ,,	163	−0·5555	152	−0·3682	210	−0·4926	190	−0·4345
Keeping quality and R.T. 37° C.	(*a*) ,,	340	0·7559	284	0·6771	105	0·6528	73	0·5086
	(*b*) ,,	387	0·7576	292	0·6679	154	0·5923	83	0·7872
	(*c*) ,,	163	0·6916	87	0·6019	210	0·3428	42	0·2610
R.T. 37° C. and R.T. 15·5° C.	R.T. 15·5° C. started at 5·0 p.m.								
	(*a*) As above	116	0·5897	97	0·4587	52	0·6565	39	0·5407
	R.T. 15·5° C. started at 10.0 a.m.								
	(*a*) As above	143	0·8454	125	0·8028	88	0·6937	62	0·7005
	(*b*) ,,	70	0·7890	59	0·7803	17	0·7891	14	0·4052
	(*c*) ,,	66	0·6147	30	0·8166	108	0·2791	13	−0·1365
Keeping quality and R.T. 15·5° C.	R.T. 15·5° C. started at 5.0 p.m.								
	(*a*) As above	—	—	116	0·7797	—	—	52	0·8096
	R.T. 15·5° C. started at 10.0 a.m.								
	(*a*) As above	—	—	143	0·8156	—	—	49	0·9097
	(*b*) ,,	—	—	70	0·8836	—	—	17	0·7697
	(*c*) ,,	—	—	66	0·8309	—	—	94	0·4724
	R.T. 15·5° C. started at 5.0 p.m. and R.T. 15·5° C. started at 10.0 a.m.								
	(*a*) As above	—	—	43	0·8324	—	—	26	0·9393
Plate count (log) and R.T. 15·5° C.	R.T. 15·5° C. started at 5.0 p.m.								
	(*a*) As above	116	−0·7062	108	−0·7430	52	−0·7165	50	−0·6759
	R.T. 15·5° C. started at 10.0 a.m.								
	(*a*) As above	143	−0·7724	81	−0·8705	49	−0·7719	30	−0·2448
	(*b*) ,,	70	−0·8127	53	−0·8051	17	−0·8512	13	−0·8340
	(*c*) ,,	66	−0·5789	58	−0·3308	108	−0·3129	82	−0·4235

On the other hand, we should expect the correlation of reduction time at 37° C. with reduction time at 15·5° C. to be at least as good as the correlation between reduction time at 37° C. and keeping quality, which is also tested at 15·5° C. (60° F.).

We have already noted the method of recording results for R.T. 15·5° C., and it follows that the available data are incomplete; consequently the values obtained may be biased. Even so, the reason for the wide range of the coefficients is not apparent but the explanation may partly lie in the fact that methylene-blue tablets can be contaminated (9) and Memo. 139/Foods (27) does not require sterilization of the stock solution.

(v) *Keeping quality and reduction time at* 15·5° C.

Hiscox *et al.* (7) showed that this relationship was very close, and Mattick (16) obtained values of 0·956 and 0·996. This series shows the highest average if we omit the last value, 0·4724, due to the inclusion of data from one laboratory where the value was especially low. The same laboratory records 0·7897 for summer samples, but the shorter keeping quality would permit less intrusion of personal bias.

(vi) *Reduction time at* 15·5° C. *started at* 5·0 *p.m. on day of production and reduction time at* 15·5° C. *started at* 10.0 *a.m. next day*

The main source of difference should be the period in the refrigerator, 5.0 p.m.–10.0 a.m. The same two laboratories contributed both winter and summer figures, the four individual coefficients ranging from 0·77 to 0·97. The number of samples does not justify any general conclusion.

(vii) *Plate count and reduction time at* 15·5° C.

As before, some very low values occur, and there is uncertainty about data for R.T. 15·5° C. In view of relationship (v) we should expect values as high as under (ii) and in fact most values are higher.

(viii) *General inferences*

As a whole there is little to choose between any pair of relationships, and our figures do not justify setting up any one test as a standard of reference.

Winter correlation of plate count and methylene-blue test

An examination of the correlation coefficients for each laboratory included in the totals given in Table 3 suggested that in the case of plate count and reduction time at 37° C. there were, on the whole, lower values in winter than in summer, even with the same workers. Further, when the mean count of a series was high, the correlation coefficient was also high.

'Indefinite' correlation coefficients are, of course, affected by the indefinite observations, because a fixed arbitrary value is substituted for the indefinite readings. These indefinite values have different weights in different series. In summer there may be a high proportion of indefinite counts, while in winter the indefinite observations are mainly in reduction times. Accordingly,

in the indefinite series the mean count, the value of r, the percentage of samples of indefinite count and the percentage of samples of indefinite reduction time were tabulated for each laboratory, and total and partial correlation coefficients between these four variates were calculated. Laboratory series totalling only a few samples were omitted, but seventeen values were obtained for winter and thirteen for summer. The results are shown in Table 4.

Table 4. *Correlation of mean count with r for plate count and* R.T. 37° C.

		r	t	n = no. in series
Summer: Def.	r_{12}	0·4075	1·91	
Indef.	r_{13}	0·6402		17
	$r_{12.34}$	−0·0634	−0·2323	
Winter: Def.	r_{12}	0·6539	3·36	
Indef.	r_{14}	0·8599		13
	$r_{12.34}$	0·6814	2·79	

Variate$_1$ = mean count. Variate$_3$ = % samples indefinite count.
Variate$_2$ = r plate count and R.T. 37° C. Variate$_4$ = % samples indefinite R.T.

As might be expected the proportion of indefinite plate counts is a significant factor in summer, while in winter indefinite reduction times affect the relation. When these variates are eliminated it is clear that in summer the level of plate count is unimportant, $r = -0{\cdot}06$, whereas in winter it is a highly significant factor, $r = 0{\cdot}68$, in determining the degree of agreement between the two tests. In winter a high initial contamination is necessary to maintain good agreement between the plate count and the reduction time, and even then the agreement is not as good as in summer. The reduction time is affected both by numbers of bacteria and by their metabolic activity in the transit period between milking time and testing time. It may be that in cold weather 'activity' is at such a low ebb that only with high initial populations are there enough organisms present to attain the potential necessary for reduction under the standard conditions of testing. The relationship here disclosed had not been previously noted, although Malcolm & Leitch (10) had shown that, in winter, milk of high bacterial content might fail to reduce methylene blue within $5\frac{1}{2}$ hr.

Other workers who have examined the effect of temperature on the reduction time of a sample have been mainly concerned with the effect of temperatures above normal atmospheric temperature.

Johns (11), using the old technique for the methylene-blue test, found that by incubating the sample for 18 hr. at 55° F. previous to testing he obtained a better agreement with the keeping quality. Hoy & Braz (12) found that temperature of storage had a great effect on the reduction time and on the ability of the test to reject milk of high plate count. Eighteen hours' storage at 70° F. was too severe. Of less severe exposures, 12 hr. at 65° F. or 18 hr. at 60° F. were found to be of almost equal effect. Pirrie (13) found that in very hot weather small 2 oz. samples taken at the farm at milking time and

exposed to transit conditions gave a worse result than samples taken from the churn on arrival at the depot and kept in the shade.

It is generally realized that exposure to high temperatures causes even samples originally good to give a short reduction time, but our figures prove that in cold weather the reduction test can yield an equally important underestimate of the bacterial population.

These findings stress the importance of sample management if justice is to be done to both consumer and producer. In summer the producer must accept some responsibility for exposure of milk to high temperatures, because milk must not only leave the farm in good order but must also reach the consumer in sound condition, while in winter there is a danger that samples containing large numbers of bacteria may pass the test if the reduction times now imposed continue to be used.

Table 5. *Samples divided and stored at* 60° F. *or in ice-chest*

Use only morning milk.

(*a*) Air temperature till 5.0 p.m., at which time plate on milk agar (*not* duplicate)

Divide

(*b*) Ice-chest 5.0 p.m.–10 a.m.	(*c*) 60° F. 5.0 p.m.–10 a.m.
Then	Then
plate singly on milk agar	plate singly on milk agar
R.T. 15·5° C.	R.T. 15·5° C.
R.T. 37° C.	R.T. 37° C.

Comparison of overnight storage in the refrigerator or at 60° F.

In the hope of securing further information on the effect of low temperature, a number of samples were specially tested (see Table 5). Each sample was divided, part being stored in the refrigerator overnight as required by Memo. 139/Foods(27), and part at 60° F. This series is therefore called 'divided samples'. The results came from the laboratories taking part in Exp. I. There was also available a series of 120 samples, from laboratory B, which had received similar treatment, except that the plate count had been made at 5.0 p.m. only instead of 5.0 p.m. and 10.0 a.m. All these were samples of morning milk taken during the winter months, October–March, and the results are shown in Tables 6–8.

A further contribution, from laboratory B, called the 'Special series', consisted of both a.m. and p.m. samples which had received 'official' treatment, i.e. morning milk was kept at shade temperature till 6.0 p.m. then placed in the refrigerator and tested by the methylene-blue reduction test at 10.0 a.m. next day; afternoon milk was kept at shade temperature till 10.0 a.m. next day. All these samples had been divided and a portion stored at 60° F. The relevant data are given in Table 9.

Table 6. *Comparison of grading*

	% samples passed or failed both tests (count and R.T. 37° C.)
Plate test 5.0 p.m., sample overnight in refrigerator, R.T. 37° C. at 10.0 a.m.	82% out of 422 samples
Sample overnight in refrigerator. Plate test 10.0 a.m. R.T. 37° C. at 10.0 a.m.	80% out of 254 samples
Sample overnight at 60° F. Plate test 10.0 a.m. R.T. 37° C. at 10.0 a.m.	84% out of 221 samples

Table 7. *Correlations, storage at* 60° F. *and in refrigerator*

Winter, a.m. samples, air temp. till 5.0 p.m.

Contributing laboratories	No. of samples	Plate count 5.0 p.m. and R.T. 10.0 a.m. (both overnight in refrigerator)	Plate count 10.0 a.m. and R.T. 10.0 a.m. (both overnight in refrigerator)	Plate count 10.0 a.m. and R.T. 10.0 a.m. (both overnight at 60° F.)
A.B.L.	394	−0·6983	—	—
C.H.D.L.	226	−0·7194	−0·8219	—
C.H.D.A.	193	−0·5902	—	−0·6741
C.H.D.	145	−0·5590	−0·6729	−0·6239

Table 8. *Effect of storage conditions on interrelationship of tests*

Detail	No. of samples	Relationship	r
1. Several laboratories (total available results)	20	Keeping quality (60° F.) and plate count (10.0 a.m.) stored overnight in refrigerator	−0·4732
	68	Keeping quality (60° F.) and plate count (10.0 a.m.) stored overnight at 60° F.	−0·5342
	20	Keeping quality (60° F.) and R.T. at 37° C. (10.0 a.m.) stored overnight in refrigerator	0·6933
	68	Keeping quality (60° F.) and R.T. at 37° C. (10.0 a.m.) stored overnight at 60° F.	0·5803
2. Laboratory B	120	Keeping quality (60° F.) and plate count (5.0 p.m.)	−0·4541
		Keeping quality (60° F.) and R.T. at 37° C. (10.0 a.m.) stored overnight in refrigerator	0·4749
		Keeping quality (60° F.) and R.T. at 37° C. (10.0 a.m.) stored overnight at 60° F.	0·5462
		Plate count (5.0 p.m.) and R.T. at 37° C. (10.0 a.m.) stored overnight in refrigerator	−0·6282
		Plate count (5.0 p.m.) and R.T. at 37° C. (10.0 a.m.) stored overnight at 60° F.	−0·7778

Table 9. *Comparison of overnight storage in refrigerator and at* 60° F.

Season	Class		Values of r: No. of samples: Including 'indef.'	No. of samples: 'Def.'	All samples	Omit indefinite counts and R.T.
Winter	Plate count (after storage at 60° F.) and R.T. (37° C.)	(i) After storage in refrigerator, i.e. official	96	53	−0·3900	−0·6209
		(ii) Stored at 60° F.	96	53	−0·1524	−0·6769
	Keeping quality and R.T.	(i) Official	96	53	0·2388	0·4368
		(ii) 60° F.	96	53	0·8670	0·6963
Summer	a.m. plate count and R.T.	(i) Official	83	62	−0·4074	−0·3813
		(ii) 60° F.	83	62	−0·6044	−0·5799
	a.m. keeping quality and R.T.	(i) Official	83	62	0·3868	0·3720
		(ii) 60° F.	83	62	0·5539	0·4522
	p.m. plate count and R.T.	(i) Official	—	16	—	−0·5967
		(ii) 60° F.	—	16	—	−0·6649
	p.m. keeping quality and R.T.	(i) Official	—	16	—	0·4174
		(ii) 60° F.	—	16	—	0·3188

Results. Divided samples

Table 6 fails to disclose any disagreement between plate count and methylene-blue test as a result of storage at 60° F. Of course, the higher temperature increased the number of samples failing to pass the tests, but the table shows that both were equally influenced by this factor, because the percentage of agreement is unaffected. Table 7 also gives no evidence that storage at 60° F. affects the relationship between the tests.

60° F. is considered to be a reasonable average room temperature in this country, and a keeping quality test at 60° F. therefore is a good measure of the commercial life of the milk, especially as domestic refrigeration is still exceptional in England. If the combined effects of conditions of production and transit to the laboratory in cold weather, followed by storage in the refrigerator overnight, and then testing, were giving a false impression of the quality of the milk, it might be possible to detect the error by correlating other methods of assessment with keeping quality (Table 8).

Unfortunately, the keeping quality had not generally been determined in the case of the divided samples and our results are indecisive (upper section of table). The lower section of the table shows a higher correlation between keeping quality and R.T. 37° C. following storage at 60° F. As the plate counts in this series were made at 5.0 p.m., there was no storage period for this test.

Special series

In the special series (Table 9) plate tests were not made till the morning after production and storage conditions could be varied. The general tendency is towards improved agreement between the methylene-blue test and either the plate count or the keeping quality test when the sample has been stored at 60° F. It may be contended that the increase in correlation with keeping quality is inevitable, since the keeping quality test also involves continuous storage at 60° F. There is practical value in so treating samples previous to testing that the results obtained are in good agreement with those of the keeping quality test, for, apart from initial standards of cleanliness, milk must not only reach the consumer in good order but must keep sweet in the house for a reasonable period. The fact that Memo. 139/Foods (27) stipulates exposure to shade temperature suggests that the standards for designated milk were intended to demand good keeping quality as well as good production.

Linearity of regression and other points

The practical importance of linearity of regression has been mentioned. When the data were plotted there were indications of possible departure from linearity when the plate count per 1 ml. was about 5000 or less. The true position was obviously much affected by the presence of indefinite results, and special calculations had to be made to allow for this. The war has prevented the completion of this work, but sufficient has been done to indicate

that the departure from linearity is only apparent and is due to variation in the proportion of indefinite results.[1]

Further examination suggested that the rate of reduction per unit change in plate count was not the same in summer and winter, the regressions based on 'definite' samples being as follows:

Summer	$X = 5{\cdot}8563 - 0{\cdot}2443\,y$	when $y = 5$ hr., $X = 4{\cdot}6438$ (44,000)
Winter	$X = 6{\cdot}0694 - 0{\cdot}2374\,y$	when $y = 5$ hr., $X = 4{\cdot}8824$ (76,000)
Summer	$Y = 11{\cdot}9732 - 1{\cdot}4663\,x$	when $x = 5{\cdot}3010$ (200,000), $Y = 4{\cdot}2003$ hr.
Winter	$Y = 12{\cdot}1984 - 1{\cdot}3375\,x$	when $x = 5{\cdot}3010$ (200,000), $Y = 5{\cdot}1083$ hr.

x = log count. y = R.T. in hours.

Wilson (2) had noted this difference between summer and winter results and Thomas & Tudor (14) give regressions for both seasons, but the latter authors do not refer to the point in their text.

It is also possible that there is a difference between morning and evening samples. Barkworth (5, 15) has shown that the keeping quality of evening milk is less than that of morning milk of similar bacterial content, and that the technical weaknesses of the test do not account for the difference. There may also be a difference if the methylene-blue test is carried out at 6.0 p.m. instead of 10.0 a.m., a course which is permissible in the case of samples of morning milk. The same difficulties arise as a result of the presence of indefinite data, but it has meantime been impossible to complete the calculations.

These points, however, have practical value where cash payments or the holding of a licence are at stake. If such differences should prove on close investigation to be statistically significant, series of samples from different producers should contain the same total number and proportion of winter and summer, and morning and evening samples. The official regulations already make an allowance of 1 hr. for the difference between summer and winter.

Effect of fat content

Wilson (2) has shown that the fat fraction of milk has an effect on the behaviour of methylene blue in milk; and an attempt was made, therefore, to see if any significant difference occurred in reduction time due to variations in fat content. Winter and summer samples were treated separately. In each section samples were classified, according to the logarithms of their plate counts, into twelve groups with a class interval of 0·3. The samples in each plate-count group were further classified into five fat-content groups, 3·0% and under, 3·5% and under, 4·0% and under, 4·5% and under, and over 4·5%. Only morning milks were used. Results were available for 263 summer samples and 122 winter samples. In each plate-count group the mean reduction times

[1] Linearity has been definitely established from other data (see 'Sensitivity experiment'). The finding of Table 4, that in winter the correlation between the two tests is lower for low counts than for high counts, seems to be due to the greater variability in reduction time for low-count than for high-count milk.

at various levels of fat content were calculated and tested for significant differences by the usual 'z' test on estimates of variance within and between samples. No significant differences were found.

This suggests that within the normal range of fat content for samples of mixed milk the fat content does not affect the reduction time.

Effect of 'coliform' content

Barkworth(5) has shown that the 'coliform' content of milk has an important effect on the keeping quality. As the reduction time is closely related to the keeping quality it is of interest to inquire to what extent the 'coliform' content of a sample affects the reduction time.

A total of 458 summer and 569 winter samples were available. Tests of significance were made as in the previous section. Plate counts were classified as before and coliform results divided into five groups, coliform absent from 1 ml., present in 1 ml., in $\frac{1}{10}$, in $\frac{1}{100}$ and in $\frac{1}{1000}$ ml. Sufficient samples were available to justify tests on seven out of eleven plate-count groups in winter and nine out of twelve groups in summer (Table 9*a*). Two winter and one summer group showed a significant fall in mean reduction time with increasing coliform content ($P=0{\cdot}05$), and in each division there was one group of doubtful significance. The remaining groups showed a trend in the same direction.

The keeping quality test is carried out at 60° F. and the methylene-blue test at 37° C., and this may affect the types of coliform organisms which proliferate.

Our results suggest that, though the effect is not marked, the presence of coliform organisms does tend to shorten the reduction time.

Summary, Experiment I

1. If the former limit of 200,000 colonies per 1 ml. be accepted, and if the treatment of the sample previous to testing is the same in both cases, the degree of agreement between the methylene-blue test and the plate count is good: 84–87 % of samples are either accepted or rejected by both tests.

2. When atmospheric temperature is low, as in winter, high correlation is obtained only when initial bacterial contamination is high.

3. If the methylene-blue test is used as a measure of keeping quality as well as of the conditions of production, samples can be stored at 60° F. before examination.

4. Reduction time for a given plate count is affected by season (winter or summer), by time of milking (morning or evening), and by age on testing.

Table 9*a*. *Effect of coliform on mean reduction time*

Figures in ordinary type denote mean R.T.; figures in black type denote number of samples

Log of plate count		Absent from 1 ml.	Present in 1 ml.	Present in $\frac{1}{10}$ ml.	Present in $\frac{1}{100}$ ml.	Present in $\frac{1}{1000}$ ml.	Significance (when tested)	No. of samples
		1	2	3	4	5		
Winter, a.m. samples								
<2·4000 (—)*	1	—	—	—	—	—	—	—
2·4001–2·7000 (2·55)	2	9·00	—	—	—	—	—	—
		1						**1**
2·7001–3·0000 (2·85)	3	8·25	—	—	8·5	—	—	—
		4			**1**			**5**
3·0001–3·3000 (3·15)	4	7·95	7·5	8·0	7·5	—	—	—
		10	**1**	**1**	**1**			**13**
3·3001–3·6000 (3·45)	5	8·21	8·93	—	7·67	—	—	—
		31	**7**		**3**			**41**
3·6001–3·9000 (3·75)	6	8·33	7·27	7·0	7·0	6·5	? 0·05	—
		32	**11**	**6**	**2**	**1**		**52**
3·9001–4·2000 (4·05)	7	7·43	6·85	7·21	7·50	7·0	N.S.	—
		22	**20**	**12**	**3**	**1**		**58**
4·2001–4·5000 (4·35)	8	7·47	6·75	6·56	1·0	3·5	0·001	—
		35	**12**	**17**	**1**	**1**		**66**
4·5001–4·8000 (4·65)	9	6·65	6·96	6·23	6·17	6·33	N.S.	—
		23	**13**	**13**	**6**	**3**		**58**
4·8001–5·1000 (4·95)	10	6·16	6·31	5·71	6·11	6·10	N.S.	—
		16	**13**	**12**	**9**	**5**		**55**
5·1001–5·4000 (5·25)	11	4·79	5·90	6·36	5·09	4·29	0·05	—
		14	**12**	**11**	**16**	**12**		**65**
5·4001–5·7000 (5·55)	12	4·17	4·25	4·90	4·72	3·06	N.S.	—
		15	**2**	**10**	**9**	**8**		**44**
No. of samples		**203**	**91**	**82**	**51**	**31**		**458**
Summer, a.m. samples								
<2·4000 (—)	1	6·66	—	—	—	—	—	—
		3						**3**
2·4001–2·7000 (2·55)	2	7·5	8·5	—	—	0·5	—	—
		2	**1**			**1**		**4**
2·7001–3·0000 (2·85)	3	7·16	6·0	8·5	—	—	—	—
		16	**1**	**2**				**19**
3·0001–3·3000 (3·15)	4	6·95	7·36	6·83	4·50	—	N.S.	—
		19	**11**	**3**	**2**			**35**
3·3001–3·6000 (3·45)	5	7·67	7·29	6·50	7·50	—	? 0·05	—
		33	**12**	**5**	**3**			**53**
3·6001–3·9000 (3·75)	6	6·95	6·97	6·0	6·8	6·5	N.S.	—
		41	**16**	**3**	**5**	**1**		**66**
3·9001–4·2000 (4·05)	7	6·21	6·70	6·93	6·5	6·63	N.S.	—
		38	**10**	**7**	**7**	**4**		**66**
4·2001–4·5000 (4·35)	8	6·38	6·67	5·50	5·56	5·25	N.S.	—
		30	**12**	**10**	**9**	**2**		**63**
4·5001–4·8000 (4·65)	9	6·20	5·59	3·13	5·38	6·0	0·05	—
		30	**11**	**4**	**8**	**4**		**57**
4·8001–5·1000 (4·95)	10	5·31	5·44	4·09	5·06	4·00	N.S.	—
		21	**12**	**11**	**9**	**6**		**59**
5·1001–5·4000 (5·25)	11	5·55	4·57	4·85	4·24	4·25	N.S.	—
		11	**15**	**17**	**15**	**16**		**74**
5·4001–5·7000 (5·55)	12	4·42	2·93	3·09	3·28	2·65	N.S.	—
		12	**7**	**11**	**17**	**23**		**70**
No. of samples		**256**	**108**	**73**	**75**	**57**		**569**

* Figures in brackets are mean log. of the group.

EXPERIMENT II. REPRODUCIBILITY

Object

The object of this section of the experiment was to test the reproducibility of results from laboratory to laboratory when differences due to transit conditions had been eliminated. In the case of the plate count Mattick *et al.* (1) have shown that reasonable reproducibility can be obtained within the laboratory, both between replicates by the same worker and also between replicates by different workers and on different samples, but that under uncontrolled transit conditions (post) it is impossible to attain agreement between different laboratories receiving subsamples from a common bulk sample.

Tests employed

The scheme of testing is given in Table 1. Three laboratories[1] participated. One of them made up subsamples from a common bulk; these were cooled to about 45° F. and sent to the other laboratories in ice boxes. The temperatures on arrival at the other laboratories were all below 50° F., and it may be assumed that variation due to differences in exposure to temperature during transit had been eliminated or was small. The fat percentage of each sample was determined and the results used to check the thoroughness of mixing. Owing to the difficulty of dealing with indefinite results, statistical analysis has been confined to samples in which they were definite.

Results of fat tests

The Gerber method was used. The Butyrometers used were either standard or had been checked against standard tubes.

An analysis of variance for samples 9–20, Table 10, shows no significant difference between the subsamples sent to the different laboratories. In the later samples the subsamples were all tested by the issuing laboratory before dispatch and the agreement was even closer. These results justify the conclusion that the samples were adequately mixed before subdivision.

Table 10. *Fat tests on subsamples. Analysis of variance*

Thirteen laboratories, thirteen samples each (single test)

	Sum of squares	D.F.	Mean square
Between samples	1·940208	11	0·1764
Between laboratories	0·007917	2	0·0040
S × L	0·168750	22	0·0077
	Total	35	

Plate counts

(i) *Difference between laboratories*

Each sample was tested in duplicate on both plain agar and milk agar, so that for each sample at each laboratory there were four counts. In this section of the experiment there were four main sources of variation, differences be-

[1] The key letters do not refer to the same laboratories as in other sections.

tween samples, laboratories, media and duplicates. After eliminating samples with indefinite counts there remained forty samples for which a complete analysis of variance is presented in Table 11.

Here the total sum of squares of deviations has been apportioned between the main sources of variation and their interactions, enabling us to see exactly what factors make a significant difference. All items involving duplicates may be combined and lead to the estimate of experimental error, 0·0288. Compared with this items 1, 3, 4, 5 and 6 are significant. The difference between samples is clearly significant even if we use the more exacting standard of comparison with S × L. However, it need not concern us further, as our object is to test differences between laboratories. Item 3 is discussed later. The direct effect of laboratory differences (item 2) is not significant, but the interactions S × L, L × M are significant. This tells us that laboratory differences vary from sample to sample and with the two media; when all samples and media are averaged this is masked so that the laboratory averages do not differ significantly. This effect must be due to differences in procedure in the different laboratories.

Table 11. *Analysis of variance. Differences between and within laboratories log$_{10}$ of plate count*

		Sum of squares	D.F.	Mean square	$\frac{1}{2}\log_e$ (×100)
1. Between samples	S	243·6553	39	6·2476	—
2. Between laboratories	L	0·0422	2	0·0211	0·3736
3. Between media	M	2·3193	1	2·3193	2·5129
4. S × L		10·8678	78	0·1393	1·3171
5. S × M		3·4001	39	0·0872	1·6927
6. L × M		0·4549	2	0·2275	1·9324
7. S × L × M		2·5205	78	0·0323	—
	Due to duplicates				
8. Between duplicates	D	0·0247	1	0·0247	0·4527
9. S × D		0·2611	39	0·0067	—
10. M × D		0·0008	1	0·0008	—
11. L × D		0·0169	2	0·0085	—
12. S × L × D 13. L × M × D 14. S × M × D 15. S × L × M × D		6·6091	197	0·0335	0·6043
16. Factors 9–15		6·8879	239	0·0288	0·5293
17. All factors due to duplicates, 8–15		6·9126	240	0·0288	0·5289
18. Total		270·1727	479	0·5640	—

(ii) *Differences between duplicates*

In an attempt to find a reason for the high value of the interaction S × L, the differences between duplicate plate counts were compared (see Table 12).

There is no significant difference between duplicates when the experiment is viewed as a whole. When each laboratory is considered individually, only in laboratory B is there a significant difference between duplicates (Table 12, lines 3, 6 and 7). Inquiries showed that this laboratory had made two sets

of dilution tubes and prepared one set of plates of each media from each set of dilution tubes. The error in this case is therefore an error between dilutions, whereas with the other two laboratories we are dealing with an error within dilutions. This error of dilution contributes of course to the error of the mean difference between the two duplicates and will increase the difference between samples in laboratory B and thus contributes to the interaction S × L.

(iii) *Differences due to media*

In Table 12 the results for milk agar have been considered separately. In all laboratories the variance of the difference between duplicates is greater when the two media are considered together. Collectively the significance just reaches the 5% level. A fortiori the difference in variance between plain agar and milk agar must be still greater. Hence milk agar gives more accurate results.

Table 12. *Differences between duplicate plate counts*

	Milk agar			Plain agar and milk agar combined		
Laboratory	B	M	N	B	M	N
1. No. of samples	40	40	40	40	40	40
2. Mean log of plate count	3·9289	3·9402	3·9852	3·8789	3·8946	3·8722
3. Mean difference	0·0381	0·0016	0·0041	0·0273	0·0172	0·0014
4. Variance of difference between single observations	0·0084	0·0046	0·0121	0·0126	0·0062	0·0130
5. S.D. of difference	0·0917	0·0663	0·1102	0·1124	0·0785	0·1139
6. S.E. of mean difference	0·0147	0·0106	0·0174	0·0126	0·0088	0·0127
7. t	2·5976	0·1333	0·2376	2·1736	1·9556	0·1085
8. Mean difference as percentage of mean count	0·9708	0·0356	0·1039	0·7042	0·4406	0·0357

(iv) *Keeping quality*

The keeping quality test (taste-and-clot-on-boiling) was carried out in accordance with the standard technique (3). Tasting is the major criterion and introduces the personal factor. Over a large number of samples a fair correlation exists between plate count and keeping quality (5), and it has been shown that a high correlation exists between keeping quality and R.T. at 15·5° C. (7, 16), but the personal error of the keeping quality test is very large. Mattick (16) found that one and the same observer might reverse an opinion reached at a previous tasting in cases where taints, probably not bacterial, occurred, and that there was no correlation between observers.

In the present experiment an analysis of variance of forty-six samples showed significant differences between laboratories.

The fact that keeping quality tests are not closely comparable between laboratories, where the difference between one palate and another plays a part, does not preclude the possibility that results in one laboratory, i.e. for one palate, are reasonably comparable. For example, twenty-six samples duplicated by laboratory B showed no differences.

Those who wish to measure keeping quality and to eliminate the high variability and personal element of the tasting test can use the R.T. at 15·5° C.

Nevertheless (except in isolated instances), tasting is the only check on the presence of original or developed off-flavours.

(v) *Reduction time at* 15·5° C.

The technique of this test has already been discussed. When results were confined to those with definite end-points, results for nine samples only, at three laboratories, remained. Further, there is a significant difference between laboratories, and the mean of laboratory B is significantly different from the mean of laboratories M and N.

However, records of eighty-nine samples which were tested in duplicate show that reproducibility within laboratories is good. Divergences occurred in only eight cases, six of which showed a difference of only half an hour, while the other two differed by 1½ and 2 hr.

The means for the three laboratories varied from 64 to 71 hr., and it is clear that at 60° F. bacterial decomposition of the milk proceeds slowly. Despite the original mixing the divergences shown by this test are, in part at least, genuine. Though the total population of each subsample was the same, the balance of types might have been different and decomposition might have followed a different course.

(vi) *Reduction time at* 37° C.

Single results for twenty samples from three laboratories are available. There is no significant difference between laboratories (see Table 13). In eighty-seven duplicated tests, differences of half an hour are recorded in only two cases, and reproducibility within laboratories is therefore very good.

For R.T. at 37° C. tubes were only inspected every half-hour, and any evaluation of reproducibility within or between laboratories is affected accordingly.

Table 13. *Analysis of variance. Reduction time* (37° C.)

Laboratories B, M and N. Twenty samples, one result from each laboratory

	D.F.	Mean square
Between samples	19	15·8533
Between laboratories	2	0·0792
S × L (error)	38	0·1757

	Means
B	48·75
M	50·00
N	49·50

Sig. diff. 2·65

These results mean that if transit hazards are eliminated, results for R.T. at 37° C. are strictly comparable from laboratory to laboratory when a careful and standard technique is rigidly observed. In other words, where standard technique is strictly observed, differences in R.T. between laboratories are not due to technical factors but are genuine differences between samples due either to different temperature hazards during transit or to different bacterio-

logical make-up of the sample. Further, replicate samples sent to different laboratories but undergoing *identical* transit temperature exposures should give results, differing only by sampling errors.

But two different samples of originally the same flora and having the same transit exposures will not necessarily give identical R.T.'s because the bacterial make-up may be different and vice versa.

Summary, Experiment II

1. When transit hazards are eliminated, and when standard technique is adhered to rigorously, the results of the plate count, the methylene-blue test and the fat test (Gerber technique) are comparable between different laboratories.

2. With the same provisos the results of the methylene-blue test at 15·5° C. and the keeping quality test are not comparable between laboratories, but results from any one laboratory are comparable with one another.

EXPERIMENT III. COMPARATIVE RELATION OF PLATE COUNT AND METHYLENE-BLUE TEST TO CONDITIONS OF MILK PRODUCTION

Object

In seeking to establish the degree to which a laboratory technique can be related to farm conditions with a view to detecting faults, some workers have tried to study individual factors. But 'clean milk' is the result of many small operations, and the effect of faults at any one stage will not necessarily be correctly ascertained when all other operations are carefully performed under controlled conditions. In other words, there is considerable interaction between a number of variables, whose value, in many cases, cannot be measured in exact units, for example, cleanliness of milkers' hands. Apart from their interdependence, each and all may vary in efficiency from one milking to another.

Score card

It is obviously necessary to use a score card to assess the value of many of these variables. The use of a score card raises two points: first, data from different experiments are unlikely to be comparable unless the same score card is used; secondly, the ability of the laboratory technique to detect changes in a given variable will depend not only on the practical effect of the variable but also on the proportion of marks allotted. Most workers are agreed that in regard to numbers of bacteria sterilization of the utensils is by far the most important factor. Barkworth (17, 18) has discussed various methods for assessing sterility of utensils, and it was an essential part of the experiment that the efficiency of sterilization should be adequately checked.

Both Wilson (2) and Nichols & Edwards (19) have attempted to correlate farm score with plate count or modified R.T., but their score cards differed from one another and our own and neither experimenter reported adequate

checks on the sterility of the utensils. The results of these workers and those presented here are not therefore strictly comparable. Stott & Barkworth (29) found that there was a high correlation between marks and laboratory results for those farms which sterilized their utensils more or less efficiently, but the correlation was low for farms which did not use steam, and where utensils and methods (apart from sterilization) were good. Nichols & Jackson (30) produced good and bad conditions artificially and obtained remarkably high correlations. It is to be noted, however, that they used very few cows.

Our samples were from morning or afternoon milk but had to reach the laboratory within 2 hr. and then be stored at 60° F. The scheme of testing is given in Table 1 and the scale of marks in Table 14.

Table 14. *Scale of marks*

I.	Sterilization		20
II.	Washing:		
	Use of cold rinse	5	
	Use of hot water	10	15
	Care and condition of brushes, cloths and stools		5
	Cooling:		
	40–45° F.	25	25
	46–50° F.	20	
	51–55° F.	10	
	56° F. and over	0	
III.	Cleanliness of udder and flanks		15
	Cleanliness of hands		5
	Milking:		
	Rejection of fore-milk	6	
	Wet or dry milking	5	
	Method of milking	4	15
	Open or covered pail		5
	Visible solid on straining cloth or disk		10
	Condition of udders (mastitis)		10
	General impression		10
			135

Two laboratories undertook this section of the experiment. Laboratory R[1] made twelve monthly visits to ten farms. Washing up and sterilization were checked by actual observation at one milking, the sample was then taken and the remainder of the score card completed at the following milking. Laboratory S[1] made three visits at monthly intervals to twelve farms. Efficiency of sterilization was assessed by rinsing the utensils with sterile saline immediately before milking time and making plate counts of the rinsings. Marks for washing up were awarded on the procedure at the close of milking.

Marks for mastitis were awarded on the results of tests with brom-cresol-purple paper. Nichols & Edwards (19) have shown that in mixed milk the presence of streptococcal mastitis does not affect the reduction time of milk. Marks were awarded at the farm by the bacteriologist, but a second and sometimes a third judge was available. Examination of the marks awarded by different judges showed that though over a series of visits two judges may come to agreement, at individual visits their opinion may differ widely.

[1] The key letters have no connexion with those in other sections.

Results

The main object of the experiment was to see which test, plate count or methylene-blue reduction test was the more sensitive to changes in production standard. Accordingly, the laboratory results were correlated with the total marks and with marks for the various sections shown in Table 14. The results appear in Table 15. These values of r suggest that neither test is

Table 15. *Correlation of plate count and reduction time with inspection marks*

	Values of r							
	Laboratory S, 35 samples		Laboratory R					
			Plate count			R.T. 37° C.		
Detail	Plate count	R.T. 37° C.	All samples (119)	Summer (49)	Winter (70)	All samples (119)	Summer (49)	Winter (70)
1. Total marks	−0·4936	−0·0312	−0·6707	−0·7126	−0·6648	0·5021	0·9330	0·3855
2. Sterilization (item I, Table 14)	−0·5835	−0·0081	−0·4662	−0·4648	−0·4762	0·4177	0·4401	0·4156
3. Cowshed and dairy (item II, Table 14)	−0·5699	0·0137	−0·5827	−0·6389	−0·5484	0·4784	0·4777	0·3428
4. Milking conditions (item III, Table 14)	−0·7301	−0·0773	−0·6349	−0·7134	−0·6330	0·4154	0·5504	0·3305
5. Items II and III. Total except for sterilization	−0·3129	−0·0360	−0·6821	−0·7298	−0·6710	0·4808	0·5574	0·3726
6. Arrival temperature	0·2948	−0·1532	0·1924	0·2227	0·1494	−0·2490	−0·2148	−0·1344
7. Atmospheric temperature	0·3213	−0·1068	0·0534	0·0281	−0·1624	−0·2594	−0·0329	−0·0518
8. Mean plate count (log)	5·0375	—	3·9902	—	—	—	—	—
9. Correlation of plate count and R.T. 37° C.	−0·3667		−0·6747 (all samples) Summer −0·5509			Winter −0·6617		

especially sensitive to changes in any of the factors measured. This may be due to insufficiently accurate marking, faulty balance of the marks scale, or a genuine incapacity of the test to reflect changes in the value of the variable. Laboratory S recorded unexpectedly low correlation between plate count and R.T. 37° C. and there is virtually no correlation in this case between R.T. 37° C. and the marks awarded. Laboratory R also obtained, almost, better correlation of marks with the plate count; the mean plate counts would suggest that the two laboratories were dealing with different grades of milk. Some of the individual values of r (plate count and R.T.) contributing to the means given in Table 3 were of the same order as that for laboratory S in this experiment.

Shade temperature was recorded at the time of sampling as well as temperature of arrival at the laboratory. The samples had to reach the laboratory within 2 hr. and were then adjusted to 60° F. The two values −0·2490 and −0·2594 for 119 samples tested by the methylene-blue reduction test (Table 15, lines 6 and 7), suggest that despite this routine, temperatures had some influence. On the other hand, when samples are divided into summer and winter series it is seen that this correlation, though in the same direction, is no longer significant.

Effect of season

In view of the findings of Exp. I on the effect of winter temperatures it was thought advisable to compare the magnitudes of the winter and summer correlation coefficients. Examination of atmospheric temperatures at time of sampling showed that the twelve visits could be divided into two periods: a summer period from May to September 1936, when on only ten occasions was the temperature below 56° F., and a winter period from October 1936 to April 1937, when on only seven occasions was the temperature above 50° F. In warmer weather any bacteria remaining on the utensils might increase more rapidly between milkings. They would probably be more active and might begin to proliferate sooner after entry into the milk. This might affect particularly the correlation with the methylene-blue reduction test because it is claimed that the reduction time is affected by bacterial activity even if unaccompanied by increase in numbers. The data are shown in Table 16, and suggest that better correlation is obtained in summer, when bacteria are more active.

Table 16. *Effect of season (laboratory R). Differences between values of r in summer and winter (from Table* 15)

	r summer $-$ r winter*			
	Plate count		R.T. 37° C.	
Detail	+	−	+	−
1. Total marks	0·0478		0·5475	
2. Sterilization		0·0114	0·0245	
3. Cowshed and dairy	0·0905		0·1352	
4. Milking conditions	0·0804		0·2199	
5. Total except for sterilization	0·0588		0·1848	
6. Arrival temperature	0·0733		0·0804	
7. Atmospheric temperature		0·1343		0·0190

* The *numerically* higher correlation coefficient has been given the positive sign.

Summary, Experiment III

Under the conditions of this experiment neither plate count nor R.T. at 37° C. is especially susceptible to any particular minor changes in production standard or method, but there is on the whole a moderate correlation with total marks as well as with individual items on a score-card basis.

The treatment of samples in this experiment was such that the effects of atmospheric temperature and arrival temperature were eliminated.

Higher correlation between marks and both plate count and methylene-blue test was obtained in summer.

SENSITIVITY EXPERIMENT

THE COMPARATIVE VARIABILITY AND SENSITIVITY OF THE PLATE COUNT AND THE METHYLENE-BLUE TEST AT 37° C.

Object of experiment—accuracy and sensitivity

Two tests such as the plate count and the methylene-blue test can be compared in various ways. For example, their relative variability may be compared by the coefficient of variation, or a correlation coefficient between them may be calculated. The validity of such procedures as these depends on the particular circumstances of the case.

A mechanical comparison of coefficients of variation between two different tests can tell us little of their relative accuracy, because the result depends on the particular measures adopted. It is obvious that the coefficients of variation of the plate count and of its logarithm, for example, will be vastly different and there is nothing a priori to tell us which to use. We have already seen, after studying the figures, that the logarithm of the plate count is the most appropriate measure to use; the standard deviation of the logarithms is therefore an appropriate measure of variability. This implies that the coefficient of variation of the original counts will be useful when it is small—for example, in dealing with replicate counts for the same sample of milk—for it is then proportional to the standard deviation of the logarithms; but when we are dealing with the distribution of plate counts in bacteriologically different milks this ceases to be the case.

Again, a correlation coefficient ceases to be an appropriate measure of association when regressions depart greatly from linearity. This is an important reason for using the logarithm of the plate count in conjunction with the actual reduction time.

The accuracy of the two tests, however, cannot be compared in any absolute sense because we do not know what they *ought* to measure. We have no absolute objective criterion of the bacterial population of a milk with which they can be compared.

'Accuracy' in far more straightforward cases than this is an ambiguous term. Take a case where there is no doubt whatever of the correct units which should be used and that these are the same for both tests. One test gives us a true mean of 100 with results ranging from 80 to 120 in 95% of cases; the other gives us a true mean of 200 with results ranging from 170 to 230. Which is the more accurate? The former absolutely, the latter relatively! It is useless to continue to argue which *is* the more accurate. We can only state the facts. Where the units of measurement are different in the two tests, no absolute comparison of accuracy is possible. If there is not agreement as to the appropriate unit of measurement in one or both tests, unprofitable controversy may arise, even about relative accuracy. In order that the coefficient of variation may be a legitimate measure of relative accuracy, the unit of measurement must be indisputable. One further condition must be satisfied.

The test must be unbiased, that is, the mean of a great many replicate determinations must give the true value. Systematic error will invalidate any measure of variability as a measure of accuracy.

The object of this experiment was to attempt to get over these difficulties by comparing the *discriminating powers* of the two tests, their *sensitivity*, that is, their ability to differentiate significantly between the individual members of a series of different samples.

Sensitivity and accuracy must also not be confused. For if one test is more sensitive than another it may be because it measures different bacteriological properties, not because it is more accurate according to some accepted definition of the term.

Samples, all from morning milk, were to be tested in quintuplicate by both plate count[1] and methylene-blue test. The methylene-blue tubes were observed every 5 min., though only inverted half-hourly, and observations were continued up to 9 hr. Six laboratories[2] contributed 169 samples. The laboratories were adequately represented, and samples at all levels of plate count and reduction time were contributed by each laboratory. This section of the experiment was carried out during the winter months.

The result by both tests for every replicate of every sample was definite, and though the data are considered under several different heads, the same 169 samples are analysed in each section.

General tables

Table 17 shows certain correlation coefficients. The importance of this table has already been indicated (p. 269). It shows what are the best units of measurement to use, the logarithm of plate count and the actual value of the R.T. Throughout this section R.T. has been expressed in minutes and not in hours and minutes.

Items 9 and 10, Table 17, the correlation between S.D. $\log_{10}$ plate count and S.D. R.T. 5 and between coefficient of variation (C.V.) for these tests, should be noted. These figures are based on quintuplicate tests, and the absence of correlation implies that the factors causing variability between replicates in the one case do not necessarily cause variability in the other test. In the case of any particular sample, high or low variability in the results by one method is no guarantee that there will be corresponding high or low variability by the other method.

The figures in item 13 are of interest. The mean C.V. of the actual plate counts is 12·4%. Wilson (2) estimates the C.V. of the actual plate count to be about 25%, derived from single plates by four workers on thirty-eight samples, or 20% based on plates counted by the same worker. Triplicate examinations by the same worker gave an average C.V. of 10%. He also says (p. 143) 'that the value of 25% is likely to be obtained only under

[1] On milk agar (3).

[2] The key letters have no connexion with those in other sections.

research conditions in a given laboratory'. Our figure of 12·4% is the average from six laboratories working under routine conditions and using the technique of the Ministry of Agriculture (3). The comparison of the C.V.'s of the logarithm of the plate count and of the actual plate count brings out clearly the fallacy of using a C.V. as a measure of accuracy. We do not advocate the use of the C.V. of the logarithm of the plate count for this purpose, but it is a useful descriptive measure of relative variability, *one* way of stating the facts.[1]

If, however, we choose to compare the C.V. of the logarithm of the plate count with the C.V. of R.T. we should, if we regarded such a comparison as a

Table 17. *Variabilities for fivefold tests on* 169 *samples, and their intercorrelation*

Correlation	Laboratory L 39 samples	M 33 samples	N 17 samples	O 20 samples	P 33 samples	Q 27 samples	All samples 169 samples
Log_{10} plate count:							
1. C.V. and mean	−0·1667	−0·2584	−0·7584	−0·3486	−0·0063	−0·3224	−0·2567
2. S.D. and mean	−0·1645	−0·0914	−0·4413	−0·1919	0·1800	−0·0299	−0·1007
*R.T. 5:							
3. C.V. and mean	−0·2332	−0·5972	−0·4333	−0·2571	−0·5048	−0·2008	−0·2203
4. S.D. and mean	0·1649	0·2364	0·2157	−0·1043	0·0750	0·5453	0·1828
Plate count (actual value):							
5. C.V. and mean	−0·2699	−0·1000	−0·3289	−0·2461	0·4093	−0·0506	−0·0424
6. S.D. and mean	0·8194	0·7631	0·9607	0·8149	0·8970	0·9544	0·6338
Log_{10} R.T. 5:							
7. C.V. and mean	−0·2646	−0·8246	−0·5063	−0·2333	−0·5533	0·1228	−0·3344
8. S.D. and mean	−0·1950	−0·6991	−0·3810	−0·1998	−0·2084	0·2237	−0·2129
9. S.D. log_{10} plate count and S.D. R.T. 5	−0·0436	−0·0231	−0·0358	−0·0785	−0·0432	−0·1094	0·0129
10. C.V. log_{10} plate count and C.V. R.T. 5	−0·0419	−0·1363	−0·3287	0·0341	−0·1492	0·1867	0·0073
11. C.V. log_{10} plate count and C.V. log_{10} R.T.	−0·0798	−0·1417	−0·3731	0·0313	−0·0940	0·0071	−0·0219
12. C.V. log_{10} plate count and C.V. actual plate count	0·7809	0·9597	0·8802	0·7716	0·8971	0·9669	0·9452
Mean C.V.:							
13. Log_{10} plate count	1·4%	1·4%	1·3%	0·6%	0·8%	3·4%	1·5%
Actual plate count	10·3%	13·7%	12·8%	5·5%	7·2%	24·8%	12·4%
Mean count:							
14. Log_{10} plate count							4·17†
Arithmetic mean							76,969‡

* R.T. 5 = R.T. observed every 5 min. † Equivalent to 14,790. ‡ Equivalent to log 4·89.

measure of accuracy, be led to believe that the plate count was more accurate than the methylene-blue test. The truth of this depends on the definition of accuracy that happens to appeal to the reader. What we are concerned to do is (1) to present the facts about variability, (2) to examine sensitivity as defined above.

Comparative variability

Table 18 shows the distribution of the C.V. There is little, if any, difference between the relative variability of the logarithm of the plate count and that of the methylene-blue test. The relative variability of the actual plate count is naturally greater (see also Table 17, item 13).

[1] For most statistical purposes the standard deviations of the logarithms of the plate count and of the actual reduction times are the appropriate measures of variability. They satisfy the criterion of independence of the mean as nearly as any simple measures can. In analysis of variance the logarithms of the plate count and the actual reduction times would be used.

A further comparison is made in Table 19 which also shows the variability of the methylene-blue test based on half-hourly observations. Although for this experiment observations were made every 5 min. the records can be adjusted so as to correspond with the routine practice of observing at half-hour intervals. This increases the variability. Average values have been calculated in three ways, and it will be noted that the C.V. of the actual reduction times is slightly greater than that of the log plate count. The data are based on quintuplicate examinations of 169 samples covering all levels of plate count and R.T., from six different laboratories scattered over England and Wales and for this reason should be carefully considered. For routine purposes it is advisable to use the figures in lines (*c*) and (*d*), Table 19.

Table 18. *Distribution of* C.V., 169 *samples. Tested in quintuplicate*

	0%	>0–1%	>1–2%	>2–5%	>7–10%	>10%
Log_{10} plate count	—	88	58	17	4	2
Actual plate count	—	4	26	62	58	19
R.T. 5	12	57	46	32	10	2

Table 19. *Variability*

			R.T. observed	
		Log of plate count	At 5 min. in min.	At 30 min. in min.
(*a*)	Mean $= \frac{\Sigma \text{ (mean of each sample)}}{169}$	4·17	422	435
(*b*) S.D.	(1) $\frac{\Sigma (\sigma \text{ for each sample})}{169}$	0·0567	7·85	—
(*c*)	(2) $\sqrt{\frac{\Sigma [S (x - \bar{x})^2]}{4 \times 169}}$	**0·0818**	**11·64**	**13·41**
C.V.	(1) $\frac{\Sigma \text{ (c.v. for each sample)}}{169}$	1·47%	2·05%	—
	(2) Based on (*a*) and (*b*)	1·36%	1·86%	—
(*d*)	(3) Based on (*a*) and (*c*)	**1·96%**	**2·76%**	**3·08%**

Although no direct evidence that the relative variability of the modified technique is less than that of the old technique is offered, it is stated (2) that the overall error of the modified methylene-blue test is 1·12%. Frayer (21), using 100 tubes per sample, showed that the relative variability of the test carried out as suggested by Wilson is less than that of the old method, and Johns (22) also claimed less variability for the modified technique. Our own figure is 3·08%.

An important factor is the occasional incidence of replicates giving widely divergent results. Wilson omitted one result in a total of thirty-six because it was irregular. Although the 100 replicates decolorized within the same quarter of an hour in twelve out of twenty samples, Frayer (21) records one sample in which two replicates were 1½ hr. less and 1 hr. more than the other 98. The two samples in Table 18 with C.V. R.T. 5 over 10% had actual values of 25·6 and 15·3%. Occasional high variabilities do occur. In other

words, just as bacteria are unevenly distributed in milk, so the reducing capacities of even such comparatively large amounts of milk as 10 ml. may at times vary substantially from one 10 ml. quantity to the next.

Variability and the mean

Table 17 shows the correlations between S.D. and the mean and between the C.V. and the mean, for both plate count (items 1 and 2) and R.T. (items 3 and 4). The regressions of the S.D. on the mean and of the C.V. on the mean have also been calculated, and tests of significance applied, leading to the following conclusions.

(i) *Plate count*

On a logarithmic basis the differences between values of the S.D. at various levels of count as calculated from the regression are not significant, and the C.V. is only slightly affected by the level of the mean.

(ii) *Reduction time*

The actual error, S.D., increases with increasing reduction time from 2·97 min. at R.T. of 15 min. to 9·26 min. at R.T. of 9 hr. and 12·85 min. at 14 hr. (tubes observed every 5 min.). However, despite this increase in the actual value of the error, the relative error decreases.

The increase of 9 min. in the actual error lends support to the widespread belief that 'accuracy' is less for milks of long reduction time, whereas the relative error (C.V.) is less than that of poor-quality milks.

Significant difference

Consideration of the standard deviation naturally leads us to inquire what difference between two samples is just significant. We have accepted a probability level of $P=0{\cdot}01$. This means that if the true values for the two milks compared did not differ, as great a difference or greater than the just significant difference would arise by chance on an average once in 100 times. We have also calculated the range of replicates. On an average, 99 out of 100 replicates would fall within the range given. If we take the anti-logarithms of the extremes of the 99% range of replicates, we get the percentage limits on either side of the true value within which 99% of the replicates will fall. The anti-logarithm of the significant difference gives the ratio of the actual counts corresponding to the significant difference which we have termed the 'significant ratio'. All these statistics are shown in Table 20. Grouping error will be discussed in the next section.

The table shows that when using single tubes and 5 min. observations, samples whose R.T.'s differ by 30 min. are not significantly different. When observed at 30 min. interval two samples whose R.T.'s differed by 30 min. would not be significantly different unless the results were the mean of triplicate tests. On the other hand, samples whose R.T.'s differ by 1 hr. are

invariably significantly different. This finding has a direct practical significance in view of the Milk (Special Designations) Order and similar schemes which award cash payments on the results of the methylene-blue test.

Table 20 brings out clearly the relevant facts about variability. In particular, it shows the impossibility of regarding any measure of variability as a measure of accuracy, unless the units of measurement are first agreed upon. The relative variability of the logarithm of the plate count is less than that of the reduction time. The relative variability of the actual plate count is very much greater. To argue from these results which test is the more accurate is idle, because on examination we find that the conclusion we arrive at depends on whether we define the accuracy of the plate count by the c.v. of the logarithm or of the actual count. The argument is completely circular.

Table 20. *Just significant differences*

	1	2	3	4	5	6
				99% range of replicates		
	s.d. of logarithm	c.v. of logarithm	Sig. diff. between two samples at $P=0{\cdot}01$	$2{\cdot}576 \times$ s.d.	Actual plate count % range	Actual plate count sig. ratio between two samples
Plate count						
1 plate at each dilution level per sample	0·0818 log	1·96%	0·2980 log	±0·2107 log	62–162%	2·0 times
2 plates at each dilution level per sample	0·0578	1·36	0·2160	0·1489	71–141	1·6
3 plates at each dilution level per sample	0·0472	1·13	0·1719	0·1216	76–132	1·4
5 plates at each dilution level per sample	0·0366	0·85	0·1333	0·0943	80–124	1·3
Reduction test						
At 5 min. observations:	s.d.	c.v.				
1 tube per sample	11·64 min.	2·76%	42·40 min.	±29·98 min.	94–106%	
2 tubes per sample	8·23	1·95	29·98	21·20	95–105	
3 tubes per sample	6·72	1·59	24·48	17·31	96–104	
5 tubes per sample	5·21	1·23	18·98	13·42	97–103	
At 30 min. observations:						
1 tube per sample	13·41	3·08	48·85	±34·54	92–108	
2 tubes per sample	9·48	2·18	34·53	24·42	94–106	
3 tubes per sample	7·74	1·55	28·19	19·94	96–104	
5 tubes per sample	6·00	1·38	21·86	15·46	96½–103½	

We have said why in our view the logarithm of the plate count is the more appropriate measure. Beyond this we would not go. The section on 'sensitivity' gives us an objective criterion of the value of the two tests in discriminating between milks of different bacterial content.

The extremes of the 99% range of replicates for the plate count (which in other statistical applications are usually called the limits of error) are not symmetrically placed about the true value. This is because the logarithms of the plate counts are normally, and therefore symmetrically, distributed (approximately), while the distribution of the actual plate counts is skew.

Grouping error

It has already been noted (Table 19) that the variability of the methylene-blue test is greater if observations take place only at 30 min. intervals instead of at 5 min. intervals. Table 20 shows that the significant difference is also increased by increasing the interval of observation to half an hour.

Wilson (2) states that the error of the methylene-blue test is 1%. However, the error of a result observed at half-hour intervals must be greater than if observed 'at frequent intervals' ((2), p. 283), even if there is no variability between replicates.

Error arising from interval of inspection is 'grouping error' and its average value can be determined by Shepherd's correction $\frac{1}{12}h^2$, where h is the interval of grouping. Thus the average increase in variance through using 30 min. in place of 5 min. intervals is $\frac{1}{12}(30^2-5^2)$ or 72·92 (min.)^2. The corresponding S.D. is 8·5 min.

The actual values from our data are:

$$\left.\begin{aligned}\sigma^2_{30} &= 179{\cdot}9\\ \sigma^2_{5} &= 135{\cdot}5\end{aligned}\right\} \text{ diff. } 44{\cdot}4, \text{ S.D. } 6{\cdot}7 \text{ min.}$$

The theoretical formula assumes that the actual end-point is equally likely to occur at any time between two intervals of inspection, and that the grouping error is independent of the reduction time. On these assumptions we have calculated that, with half-hourly observations, the error in the difference between two single tubes, due to grouping only, will exceed 27 min., in the long run, in 1% of cases.

Sensitivity tests

The conditions under which variability is a measure of accuracy have already been discussed. In the case of the two tests with which we are concerned we cannot measure accuracy because we have no absolute standard to which to refer; we can only compare the relative variabilities on the common basis of C.V.

At the outset we stated that this experiment was designed to examine the comparative ability of the tests to discriminate between samples; this discriminatory power, or 'sensitivity', is, we think, a suitable basis for final judgement.

We are not aware that this point has been examined before by any of the workers who have attempted to ascertain which test is the best measure of the bacteriological quality of milk.

The following argument will explain the points involved in distinguishing between accuracy, variability, and sensitivity.

Accuracy and sensitivity

In past work, the idea underlying the use of the term 'accuracy' with reference to these tests has been that accuracy should measure the degree of agreement between replicate determinations. The extent to which the results depend on the scale of measurement adopted was inadequately appreciated, a point already discussed. When the scale had been settled, if a test, plate count or R.T., gave the same answer for all replicates it was regarded as completely accurate. This conclusion is only true in the absence of bias. Bias implies that the mean of a very large number of determinations does *not* tend

to be equal to the true value; where it exists no measure of variability alone can be a measure of accuracy. Neglect of this consideration would lead to the absurd conclusion that a test which gave the same answer for all samples of all milks would be '100% accurate', whereas it would obviously be useless. Again, a test may give different answers to replicates just because it is capable of reflecting minute changes in the make-up of the subsamples on which the replicate determinations are made. The error of replicate plate counts (c.v. = 2% of the log count) is largely due to the sampling variation in the distribution of bacteria in the dilution, not because the laboratory technique is giving the wrong answer for the individual plates. If one plate gives twenty-five colonies and another thirty-two it is very probable that this is the truth.

Wilson (2) examined the plate count stage by stage; errors due to graduation of pipettes and measurement of the dilutions are of the order of 1%; the depth of agar affects the number of colonies, but this is controlled by adding a fixed volume of medium to a Petri dish of standard size; with suitable incubators variations in count due to temperature fluctuations within the incubating chamber can be rendered very small. The only serious factor appears to be the error arising in the actual enumeration of the colonies (7%). Barkworth (23) gives 3·3% for plates with low numbers of colonies and 2·7%[1] for high-count plates. The small size of these errors suggests that the differences between replicate plates are not errors due to manipulation, but perhaps a genuine difference due to uneven distribution of bacteria (i.e. sampling error together with other sources of unevenness such as the breaking of clumps). In other words, we have a mechanism, sensitive to small changes in bacterial population. Because bacteria are unevenly distributed and because we can only count the population of a very small portion of milk on one plate, we get variations from plate to plate, regarded as 'error'. The count of milk of average quality is assessed on a plate of $\frac{1}{100}$ or $\frac{1}{1000}$ ml. milk, and there is consequently a variation, due to sampling, in the number of bacteria from one portion of $\frac{1}{100}$ ml. to another. The plate count is not inaccurate (in the sense of giving a wrong number of colonies) but hypersensitive (in the sense of detecting sampling variations between plates).[2] A better estimate of the average population of the bulk will only be obtained by replication.

It has been claimed ((2), p. 143) that the methylene-blue test is more 'accurate' than the plate count, but accuracy, measured by agreement between replicates, is not the only desideratum and may even give a false impression. The question, therefore, is to consider not only the 'accuracy' of both tests as usually measured (i.e. the relative variability of replicates), but also their comparative discriminating power (sensitivity), or ability to differentiate between samples.

[1] The figure 8·1% in the reference is a misprint and should be 2·7% as given here.

[2] Still another use of the terms 'accuracy' and 'sensitivity' will be noted here. It is, however, clearly indicated.

It will be clear from the argument above that an 'inaccurate' but sensitive test might, on the basis of replication, distinguish between samples, while an 'accurate' but insensitive test might fail to show any significant difference.

Regression equations

We shall shortly make use of the regression equations to test comparative sensitivity. We have available the mean plate count from quintuplicate tests and the mean R.T. from five tubes observed at 5 min. intervals for 169 samples covering all levels of plate count and of reduction time. These provide a sound basis for calculating the regressions. In order that their linearity might be tested the data were grouped. The class intervals were 30 min. for R.T. (y), and 0·2 for $\log_{10}$ plate count (x), giving a (22×22) table with entries in 21 arrays for x and 19 arrays for y. Correlation of $\log_{10}$ plate count with reduction time gave the following equations:

Regression of plate count on reduction time:

$$X = 5{\cdot}77 - 0{\cdot}0038y; \qquad (1)$$

Regression of reduction time on plate count:

$$Y = 898 - 114x; \qquad (2)$$

where $x = \log_{10}$ plate count, $y =$ reduction time in min.; $X =$ mean log count for given reduction time, $Y =$ mean reduction time for given log count.

From equation (1): The average fall in log plate count corresponding to a rise of 30 min. in reduction time is 0·114 (or an average fall in the ratio of 1·3 to 1 in the actual count).

From equation (2): The average fall in reduction time corresponding to a rise of 1·000 in log plate count (a tenfold increase in actual count) is 114 min. (1 hr. 54 min.).

No significant departure of either regression from linearity was found by the usual statistical test ('z' test).

The relation between reduction time and actual plate count is shown in Fig. 1 and is definitely non-linear.

Corresponding values of plate count and reduction time

The regression equations enable us to calculate the mean reduction times corresponding to a given log plate count and vice versa. The reader will note that whereas the average count (geometric mean) corresponding to a reduction time of 1 hr. is 346,000, the count for which 1 hr. is the average reduction time is 22,400,000. To see why this is so, it must be understood that if corresponding individual values of log count and reduction time are plotted we get not a curve but a scatter diagram. Consequently, there are two regression lines, one giving the average log count for given reduction time, the other the average reduction time for given log count. These cut one another at the point which corresponds to the mean log count and the mean reduction time, and diverge considerably from one another at the ex-

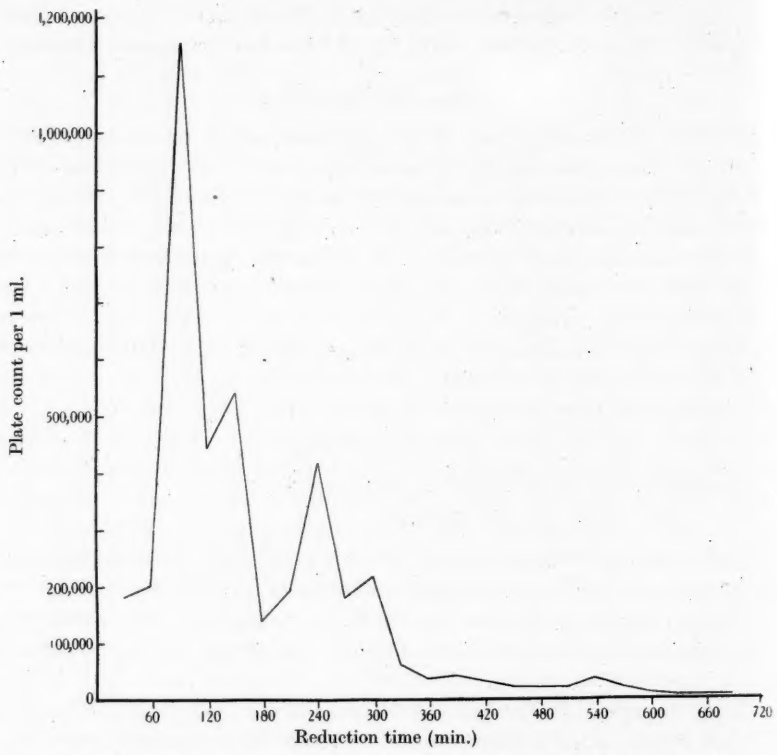

Fig. 1. Mean arithmetic plate count at given level of reduction time (169 samples). The irregularity in the first two points is due to the small number of samples examined.

Table 21. *Average values of plate count for given reduction time and vice versa*

$X = 5{\cdot}77 - 0{\cdot}0038y$			$Y = 898 - 114x$		
When R.T. is in hr.	Mean $\log_{10}$ plate count will be	Approx. arithmetic equivalent	When $\log_{10}$ plate count is	Approx. arithmetic equivalent	Mean R.T. will be in hr.
9	3·72	5,200	3·14	1,400	9
8	3·95	8,900	3·67	4,700	8
7	4·18	15,000	4·19	15,500	7
6	4·40	25,000	4·72	52,500	6
5½	4·52	33,000	4·98	95,500	5½
5	4·63	42,000	5·25	178,100	5
4½	4·74	55,000	5·51	323,600	4½
4	4·86	72,400	5·77	589,000	4
3	5·09	123,000	6·36	1,995,000	3
2	5·31	204,000	6·82	6,600,000	2
1	5·54	346,000	7·35	22,400,000	1

$x = \log_{10}$ plate count. y = reduction time in minutes.

tremes of the range. Hence arises the fact that the average log count for a reduction time of 1 hr. is 5·54 while 1 hr. is the average reduction time corresponding to a log count of 7·35 (see Table 21).

Sensitivity by regression

One method of testing comparative sensitivity is to use the findings of Table 20 in conjunction with the regression equations. Table 20 shows that the just significant difference in log plate count between two samples (single plates) is 0·2980. If the difference in reduction time which corresponds to this value is greater than the just significant difference in R.T., the R.T. may be said to be the more sensitive test. The correspondence may, however, be of two kinds. We may calculate from equation (1) the reduction time for which 0·2980 is the mean log count or from equation (2) the mean reduction time corresponding to a log count of 0·2980. The first value is 78 min., the second 34 min. The just significant difference in reduction time is 42·40 min. for R.T. 5. This is less than 78 but greater than 34 min., so on this basis there is little difference in sensitivity between the two tests. We may, however, compare 42·40 with the mean of 78 and 34, that is with 56 min., which shows an advantage with R.T. 5 over the plate count.

Similarly, we find an advantage with R.T. 30 over the plate count. Tables 22 and 23 show this and similar calculations for two, three and five replicates, leading to the same conclusion.

We might, however, start from the just significant difference in R.T. 5, 42·40 min. The corresponding difference in log count is 0·1611 from equation (1) and 0·3719 from equation (2), and the just significant difference in plate count, 0·2980, is between the two. However, it exceeds 0·2665, the mean of 0·3719 and 0·1611. So again we find an advantage with R.T. 5 over the plate count.

With R.T. 30 the just significant difference is 48·85 min. The corresponding difference in log count is 0·1856 from equation (1) and 0·4285 from equation (2). Again 0·2980 is between the two but less than their mean (0·3071), showing a very slight advantage to the plate count over R.T. 30. Tables 22 and 24 show this and similar calculations for two, three and five replicates, leading to the same conclusion.

We conclude from the regressions that sensitivity is in the order R.T. 5 > R.T. 30 > plate count, but the difference between the last two is small.

The 't' test and sensitivity

't' is the ratio of the mean difference between two samples to an appropriate estimate of its standard error. Its sampling distribution on the hypothesis that the samples come from the same homogeneous normally distributed material and that the true means are therefore identical, has been tabulated by 'Student' and R. A. Fisher. From one of these tables we can obtain the values of 't' which, on the hypothesis in question, would only be exceeded

Table 22. *Just significant differences and corresponding values by regression*

x = log plate count. X = mean log count for given reduction time.
y = R.T. (min.). Y = mean reduction time for given log count.

		Regression of count on R.T. $X = 5{\cdot}77 - 0{\cdot}0038y$		Regression of R.T. on count $Y = 898 - 114x$	
		X	y	x	Y
		(1)	(2)	(3)	(4)
		Log count	Reduction time for which (1) is the mean log count min.	Log count	Mean reduction time corresponding to (3) min.
Plate count:	1 replicate	**0·2980**	78	**0·2980**	34
	2 replicates	**0·2106**	55	**0·2106**	24
	3 replicates	**0·1719**	45	**0·1719**	20
	5 replicates	**0·1333**	35	**0·1333**	15
		(5)	(6)	(7)	(8)
		Mean log count corresponding to (6)	Reduction time min.	Log count for which (8) is the mean R.T.	Reduction time min.
R.T. 5:	1 replicate	0·1611	**42·40**	0·3719	**42·40**
	2 replicates	0·1139	**29·98**	0·2630	**29·98**
	3 replicates	0·0930	**24·48**	0·2147	**24·48**
	5 replicates	0·0721	**18·98**	0·1665	**18·98**
		(9)	(10)	(11)	(12)
		Mean log count corresponding to (10)	Reduction time min.	Log count for which (12) is the mean R.T.	Reduction time min.
R.T 30:	1 replicate	0·1856	**48·85**	0·4285	**48·85**
	2 replicates	0·1312	**34·53**	0·3028	**34·53**
	3 replicates	0·1071	**28·19**	0·2473	**28·19**
	5 replicates	0·0831	**21·86**	0·1918	**21·86**

Values in heavy type are just significant differences, see Table 20.

Table 23. *Sensitivity by regression*

	Calculated significant differences in R.T., based on S.D. Observed every		Significant differences by plate count expressed as R.T. (via regression equation)
Replicates	5 min. in min.	30 min. in min.	in min.
1	42·40	48·85	56·0
2	29·98	34·53	39·5
3	20·48	28·19	32·5
5	18·98	21·86	25·0

Table 24. *Sensitivity by regression*

		Significant differences by R.T. expressed as plate count (via regression equation). Observed every	
Replicates	Calculated significant differences in plate count, based on S.D.	5 min.	30 min.
1	0·2980 log	0·2665 log	0·3071 log
2	0·2106	0·1890	0·2179
3	0·1719	0·1538	0·1772
5	0·1333	0·1193	0·1375

in, say, 20, 5, 1 or 0·1% of cases. If the calculated value exceeds an accepted standard (the tabulated 5% value is usually taken) the two means are said to differ significantly. There *are*, of course, significant differences between these 169 samples, and that test which leads to the greater number of significant values of '*t*' will be detecting differences more easily and in consequence be more sensitive.

This has been examined in two ways. First, the number of significant steps (see below) when all samples are arranged in various orders has been ascertained, and secondly, a comparison has been made of the distribution of '*t*' values, when all possible pairs (in a limited number of samples) are tested.

Significant steps

If samples are arranged in ascending order of log plate count, consecutive samples will not necessarily be significantly different, but possibly the first and fourth will differ; proceeding from the fourth we can find the next sample which is significantly different from sample 4, and so on until we have reached the 169th sample. Thus we can establish a number of steps, each step taking us to the nearest significantly different sample (in our list). Having found the two samples which form the 'step' we can test if they are also significantly different by R.T. 5 or R.T. 30. The results appear in Table 25. (For this purpose the 5% significance level was used.)

Table 25. *Significant steps*

In order of	No. of 'sig. steps'	Same samples tested by
Log plate count	37	R.T. 5, 7 steps give N.S.
R.T. 5	46	Log plate count, 4 steps give N.S.
R.T. 30	21	Log plate count, 2 steps give N.S.

N.S. = no significant difference.

These results suggest that sensitivity is in the order R.T. 5 > plate count > R.T. 30. R.T. 30 is clearly the least sensitive; not only are the steps wider but 90% of the corresponding differences are detected by the plate count. The steps are somewhat wider for the plate count than for R.T. 5; on the other hand, the plate count detects 90% of the 'R.T. 5 steps', while the R.T. 5 only detects 80% of the 'log plate count steps'.

Testing all possible pairs of samples

(*a*) *Groups of ten consecutive samples*

The 169 samples were arranged in order of mean log count, and groups of ten consecutive high-grade, ten consecutive medium-grade and ten consecutive low-grade samples were selected. '*t*' tests were made between all possible pairs of samples in each group of ten (forty-five pairs) using log count, R.T. 5 and R.T. 30. Next, the 169 samples were arranged in order of mean R.T. 5, three similar groups were selected, and again the significance of the

differences between all possible pairs of samples was tested using all three determinations. In the first case, we have samples of nearly the same plate count but possibly greater differences in R.T., and in the second case the position is reversed. By mean R.T. 30 the order of samples is essentially the same as by mean R.T. 5.

Section 1 of Table 26 gives the results. The order of sensitivity is R.T. 5 > plate count > R.T. 30, though the former two do not differ significantly.

Table 26. *Distribution of 't' tests*

			Value of 't' which, in sampling homogeneous material, would be exceeded by chance in					
	't' test applied to	Indeterminate	More than 20% of samples	More than 5% but not more than 20%	More than 1% but not more than 5%	More than 0·1% but not more than 1%	0·1% and under	Total
1. All possible pairs from groups of consecutive samples (6 × 45)	Log_{10} plate count	—	58	24	19	23	146	270
	R.T. 5	1	51	27	23	27	141	270
	R.T. 30	22	60	29	26	14	119	270
2. All possible pairs, 10 consecutive samples by actual mean count (3 × 45)	Log_{10} plate count	—	44	27	17	18	29	135
	Actual count	—	48	26	14	19	28	135
3. All possible pairs, 15 samples, random selection	Log_{10} plate count	—	5	3	5	—	92	105
	Actual count	—	8	1	3	9	84	105
	R.T. 5	—	2	2	4	4	93	105
	R.T. 30	4	6	—	7	1	87	105

Three groups of ten consecutive samples were also selected in order of actual mean count. The order of the 169 samples is of course essentially the same by actual mean plate count or by mean log count, but there is a difference in the distribution of 't' according as the actual or logarithmic values are used. This difference is slight and non-significant. The interest of this result is in showing that the *replicate* counts are sufficiently close together for the actual values to be used in place of their logarithms without affecting the *general* result of the sensitivity tests. Nevertheless, the use of actual values for a *particular* pair of samples may be misleading if the levels of count of the two samples differ widely.

(*b*) *Fifteen samples selected at random*

Finally, using Tippett's random numbers, fifteen samples were selected at random; 't' tests were again applied to each possible pair of samples, using log plate count, actual plate count, R.T. 5 and R.T. 30. Section 3 of Table 26 gives the corresponding distribution of 't'. R.T. 5 appears slightly more sensitive than the other two, but there are no significant differences in sensitivity.

Conclusions

Table 27 summarizes the findings of the experiments on accuracy and sensitivity. The methylene-blue test *when observed every* 5 *min.* is probably the most sensitive of the tests examined.

The plate count is probably not quite so sensitive as the R.T. 5, but, on the whole, superior to the methylene-blue test with only half-hourly observations.

Table 27. *Order of decreasing sensitivity*

	Plate count	R.T. 5	R.T. 30
By regression equations	3	1	2
't' tests (significant steps)	2	1	3
Groups of 10 samples	2	1	3
15 random samples	2	1	3

Summary of sensitivity experiment

1. Variability is not necessarily a measure of 'accuracy'. In order that the coefficient of variation may be a legitimate measure to use in the comparison of the relative accuracies of two tests we must know what the tests *ought* to measure, the units of measurement must be indisputable and the tests must be unbiased. The first two conditions are not fulfilled by the plate count and methylene-blue test.

2. The mean coefficient of variation of the logarithm of the plate count is less than that of the reduction time, even when observations take place every 5 min.

3. The significant differences between two samples in single tests (one plate or one tube) are 0·2980 in the logarithm of the plate count (corresponding to a ratio of about 2·0 in the actual plate counts), 42·40 min. in the methylene-blue test if observed every 5 min. and 48·85 min. if observed only every half-hour.

4. In the methylene-blue test, observing only every 30 min. involves a grouping error, the effect of which has been calculated.

5. The only objective criterion of comparison between the plate count and the methylene-blue test, that we have, is constituted by discriminatory tests of their ability to distinguish significantly between members of the same set of samples of milk.

6. Such 'sensitivity' tests were made by calculations based on the regression equations and by 't' tests, giving the order R.T. 5 > plate count > R.T. 30.

ADDENDUM I. THE NORMALITY OF THE DISTRIBUTIONS OF PLATE COUNTS

BY H. B. BESCOBY

Introduction

In the statistical treatment of bacteriological counts of milk, it is sometimes assumed that replicate counts are distributed in a Poisson distribution and not in a normal distribution. When the mean number of colonies is twenty or more there is little difference between the two assumptions, since the Poisson distribution tends to the normal form as the mean increases. Usually both assumptions are incorrect. Values of χ^2 for small sets of parallel plates

are often too large to be in accordance with a Poisson distribution, and inspection of larger sets has shown that the distribution of actual counts is not normal (6). In many calculations the replicate counts have been transformed into logarithms, and it would be possible for these to conform with a normal distribution when the original counts do not.

The data available have been examined statistically by transforming the actual counts into three decimal figure logarithms and testing for normality. The data had been collected from six laboratories throughout the country. Each laboratory was asked to make five-fold tests by both plate test and methylene-blue test, the latter to be observed every 5 min. and every 30 min. These results formed suitable data for normality tests and 169 samples were examined each with five subsamples, a total of 845 subsamples.

The normality test

The statistical examination consisted of the normality test given by Fisher (28).

It was first shown by Karl Pearson and is well known that for a normal distribution

$$\sqrt{\beta_1}=\frac{\mu_3}{\mu_2^{\frac{3}{2}}}=0, \quad \beta_2=\frac{\mu_4}{\mu_2}=3,$$

where the μ's are moments about the mean. Departures of $\sqrt{\beta_1}$ from zero indicate skewness; if the departure is positive the longer tail of the distribution is to the right giving a positive skewness, a negative departure gives a negative skewness.

If β_2 is greater than 3 (or $\beta_2-3>0$) the distribution has a higher top and longer tail than the normal distribution (when the distributions are plotted so that the total frequency is represented by the same area in each case). This form of departure from normality is called 'kurtosis'. If β_2 is less than 3 the contrary is the case. Estimates of $\sqrt{\beta_1}$ and β_2 and their standard errors can be made from the observed data. If the sample is large it is adequate to calculate the same functions of the sample moments, but if it is small it is advisable to use the improved estimates given by Fisher:

$$g_1=\frac{k_3}{k_2^{\frac{3}{2}}}, \quad g_2=\frac{k_4}{k_2^2}.$$

g_1 is an estimate of $\sqrt{\beta_1}$ and g_2 an estimate of (β_2-3) both of which are zero in a normal distribution. Departures of g_1 and g_2 from zero indicate skewness and kurtosis and may be tested for significance by comparison with their standard errors.

In the above formulae, k_1 is the sample mean; k_2, k_3, k_4 are unbiased estimates of the quantities μ_2, μ_3, $\mu_4-3\mu_2^2$ respectively. Using the formulae provided by Fisher their values were calculated for each sample of five observations, the logarithms of the plate counts and the actual reduction times being used. From the k's, g_1 and g_2 were found.

Logarithm of the counts

In this investigation of 169 samples taking the logarithm of the counts to three figures the following results were calculated:

Logarithms of plate counts

Laboratory[1]	No. of samples	Σg_1	$\bar{g}_1$	S.E.	Σg_2	$\bar{g}_2$	S.E.
L	39	12·171	**0·31**	0·15	44·239	**1·13**	0·32
M	33	−12·151	**−0·37**	0·16	19·749	0·60	0·35
N	17	0·291	0·17	0·22	0·963	0·57	0·49
O	20	−4·130	−0·21	0·21	−6·165	−0·31	0·45
P	33	10·832	**0·33**	0·16	−9·351	−0·28	0·35
Q	27	−5·159	−0·19	0·18	−2·087	−0·77	0·39
Total	169	1·854	0·011	0·072	47·348	0·281	0·154

Values exceeding twice their standard error are in black type.

In a normal distribution g_1 and g_2 should be zero. The general means indicate departure in a positive direction, g_1 being 0·16 times its standard error and g_2 1·83 times. These are not significant departures. Laboratories L, M and P show possibly significant departures from symmetry, but these are small and not of the same sign. L shows a definitely significant positive value of g_2, but even this is not large. We can conclude that the observed distribution of the logarithms of the counts differs but slightly from a normal distribution.

The fact that the logarithms of the counts conform to a nearly normal distribution while the original counts do not, enables methods of statistical interpretation appropriate to the normal distribution to be used if logarithms are employed. Among these is analysis of variance, provided that the further condition, that the variance is independent of the mean, is satisfied.

Reduction time

The actual 5 and 30 min. reduction times were dealt with by exactly the same methods with the following results:

Reduction time—5 min. readings

Laboratory	No. of samples	Σg_1	$\bar{g}_1$	S.E.	Σg_2	$\bar{g}_2$	S.E.
L	39	6·048	0·16	0·15	8·947	0·23	0·32
M	33	6·443	0·20	0·16	−19·647	−0·60	0·35
N	17	−2·257	−0·13	0·22	10·313	0·61	0·49
O	20	2·731	0·14	0·21	−14·224	−0·71	0·45
P	33	29·622	**0·90**	0·16	39·197	**1·19**	0·35
Q	27	−8·800	−0·33	0·18	−11·767	−0·44	0·39
Total	169	33·787	**0·200**	0·072	14·219	0·084	0·154

Values exceeding twice their standard error are in black type.

The mean value of g_2 is not significantly different from zero, but g_1 is 2·78 times its standard error, which indicates significant positive skewness.

[1] The key letters refer to the same laboratories as in the 'Sensitivity' section.

The departure is only significant in one laboratory and is not consistent in direction from one laboratory to another. In amount it is not sufficiently large to affect the use of normal distribution theory for statistical purposes.

Reduction time—half-hourly readings

Laboratory	No. of samples	Σg_1	$\bar{g}_1$	S.E.	Σg_2	$\bar{g}_2$	S.E.
L	39	3·351	0·08	0·15	6·603	0·17	0·32
M	33	2·642	0·08	0·16	13·667	0·41	0·35
N	17	−1·629	−0·10	0·22	18·667	**1·10**	0·49
O	20	5·150	0·26	0·21	0·391	0·02	0·45
P	33	2·236	0·07	0·16	−8·333	−0·25	0·35
Q	27	−7·455	−0·28	0·18	16·397	0·61	0·39
Total	169	4·295	0·025	0·072	47·392	0·280	0·154

Values exceeding twice their standard error are in black type.

The value of g_2 for one laboratory just exceeds twice its standard error, otherwise none of these ratios is significantly greater than zero; therefore half-hourly readings in the reduction test form a nearly normal distribution.

Conclusions

The distribution of the logarithms of the counts, and of actual 5 min. and half-hourly reduction times from six laboratories, indicate that methods applicable to normal distributions may be used for the statistical examination of data of this kind. Data from individual laboratories sometimes resulted in significant departure from normality. This departure was in no case consistent for all samples at any laboratory, and the departure was not sufficiently large to indicate that normal distribution methods of statistical interpretation could not be used.

Addendum II. χ^2 values for plate-count replicates

The values of $\chi^2 = S\,(x-\bar{x}^2)/\bar{x}$ (where x = actual plate count) are frequently calculated for sets of parallel plates prepared from the same dilution tube, in order to test whether the variance of replicates is inconsistent with that to be expected in the ideal case when replicate counts have a Poisson distribution. For milk the values of χ^2 are usually greater than expected, and in this respect our findings confirm those of Wilson (2). Table 28 shows the values of $\sqrt{(2\chi_1^2)} - \sqrt{(2n-1)}$ for six separate laboratories and for all samples together. Here χ_1^2 is the total value of χ^2 from the set of samples considered, and n the corresponding numbers of degrees of freedom. For the values of n under consideration $\sqrt{(2\chi_1^2)}$ may be taken as normally distributed about $\sqrt{(2n-1)}$ with unit S.D.

The conditions under which replicate counts would follow a Poisson distribution are

(1) The organisms must be randomly distributed throughout the dilution.
(2) Each organism must have the same opportunity for development.

(3) The development of each organism must be independent of that of other organisms present.

(4) Each organism must give rise to one colony.

Departure from these conditions usually results in χ^2 values being excessive; probably the most influential cause of this is the irregular breaking up of clumps. Competition tends to lead to χ^2 values that are too small. The latter factor may be the cause of the subnormal values in laboratory O, while the former seems to have been most influential in laboratory Q. There are thirteen values of χ_1^2 which are quite abnormally large. If we omit these we are left with 156 samples for which the values of $\sqrt{(2\chi_1^2)} - \sqrt{(2n-1)}$ is 17·31,

Table 28. *Values of* $\sqrt{(2\chi_1^2)} - \sqrt{(2n-1)}$

(Number of samples in brackets)

	Laboratory						All samples
	L	M	N	O	P	Q	
Plate count, actual:							
(*a*) all samples	16·93 (39)	27·05 (33)	10·41 (17)	−3·66 (20)	6·35 (33)	54·96 (27)	58·05 (169)
(*b*) omitting abnormally high values	4·95 (37)	15·01 (31)	—	—	1·72 (32)	9·08 (19)	17·31 (156)
(*c*) values of χ^2 omitted	110·394 242·017	95·080 369·104	—	—	98·34	139·456 161·266 161·883 201·243 278·656 296·362 369·426 587·282	—

with 624 degrees of freedom. Wilson, considering different dilutions separately, found values ranging from 3 to 22 with 173–178 degrees of freedom. When the values of χ^2 are significantly in excess of expectation, the value of $\sqrt{(2\chi^2)} - \sqrt{(2n-1)}$ increases with n, because the more data there are available, the more clearly is the significance brought out.

These excessive values of χ^2 could have been anticipated from our previous results. For we have found that the S.D. of log count is approximately independent of the mean. This implies that the C.V. of the actual count is approximately independent of the mean, as has also been verified. Hence the χ^2 values must be excessive (at any rate when the mean is sufficiently large), for if the variance of replicates were equal to the mean m, the C.V. $100/\sqrt{m}$ would decline with m. Thus the values of χ^2 are consistent with our previous results and in no way invalidate the comparisons of sensitivity which have been made.

ADDENDUM III. COMPARISON OF PLAIN AND MILK AGAR

Variability

Several workers have shown that milk agar gives higher plate counts than plain agar (2, 7, 24), and Barkworth (8) has shown that the increase in count is statistically significant. Safford & Stark (25) compared 0·5 and 2·0% milk agar,

and Barkworth (26), discussing the differences in composition between certain milk-agar media, suggested that the increase due to 2% over 0·5% was statistically significant.

The first series considered was tested in 1936 as part of Exp. I, and the analysis was made in 1937 though publication has been delayed till completion of the entire report. The samples were tested in duplicate on milk agar (3) and plain agar (4), and an analysis of variance of the difference between duplicates shows a significant difference in favour of milk agar (Table 29).

Table 29

(Number of samples in brackets)

Count assessed	Variance of difference between duplicates (log count basis)	
	Plain agar	Milk agar
(*a*) Wilson's method ((2), p. 148)	0·01730 (116)	0·01082 (118)
(*b*) Bull. 46 (3)	0·01609 (116)	0·009274 (118)

In a later series two laboratories compared milk agar (3) and yeastrel agar (27). Single tests were made, but plates of both media were incubated at 32° C. as well as at 37° C.

Comparison of yeastrel and milk agar

Laboratory V, twenty-eight samples pasteurized milk

Analysis of variance showed that the temperature of incubation was a significant factor. There were significantly higher counts at 32° C. on yeastrel but not on milk agar (27, 3).

When compared at the same temperature there was no significant difference in mean count between these two media, but the variability of the difference between media is significantly greater at 37° C.

Laboratory W, thirty-two samples pasteurized milk

These samples were only incubated at 37° C., and in this series milk agar gave significantly higher counts, the mean increase being 0·0644 log.

Addendum IV. Method of counting

Differences due to method of assessing final count

By the technique of the Ministry of Agriculture (3) the reported count is based on only one plate out of three. This is from the lowest dilution in which there is a plate with between 30 and 300 colonies, i.e. the highest countable plate is selected. Wilson (2) suggested the range 30–500 colonies, and proposed that the arithmetic average of the reported results be taken whenever more than one plate fell between these limits. The Ministry of Health (27) give no directions on the method of assessing the count. Against these proposals (2)

it may be advanced that they provide an additional complication for probably very little gain in accuracy, and that only in a limited number of cases can this method be applied. Moreover, if two dilutions gave countable plates that from the lower dilution would have more colonies and this in itself would tend to greater accuracy in counting (2, 23).

An analysis of variance was made of twenty-two samples to which this method of counting was applicable. The samples were tested in duplicate on milk agar (3), and in twelve cases the replicates gave the same final count by both methods, but there was a significant difference between the variabilities of replicates, the differences being greater by the new method. This series contained 77% high-count samples—over 4·0 log.

An analysis of fifty-one other results from five different laboratories failed to disclose any significant difference in variability, but when twenty-four of these samples with counts over 4·0 log were combined with the original twenty-two, the variability between replicates remained significantly greater by the new method of assessing the final count.

A third series was obtained from the results of Exp. II. In this series there was again no significance in variability, but the counts by the Ministry of Agriculture's method were slightly but significantly greater with a mean difference of 0·0147 log. This series contained ninety-one samples, all under 30,000 per ml.

The general inference is that the method of assessing final count does not affect the result.

ADDENDUM V. ERROR OF THE PLATE COUNT

In a simple technique such as the methylene-blue test where only one tube of milk is involved, 'error' in the sense of difference between replicates is easily understood. The technique of the plate count is more complex.

The normal practice is to withdraw a single test portion of 1 ml. from the sample, to prepare a single set of dilution tubes and, if any replication is desired, to prepare several plates from each dilution tube. The difference between replicates is an error within dilutions. Even if error is based on reported plate counts this statement still holds good because it would be exceptional for reported counts to involve plates at two different dilution levels.

Irregularities of distribution of bacteria in the milk can only be checked by taking several test portions from the milk and preparing sets of dilutions from each test portion (of 1 ml.). We can also prepare replicate sets of dilution tubes, for each portion, for the 1/100 dilution and onwards, but we cannot make replications of the 1/10 dilution, only one such dilution can be prepared per test portion.

Error between replicate portions is true sampling error combined with errors in technique, i.e. errors in calibration of glassware and errors in mani-

pulation due to measurement of the dilution blanks and of the amounts transferred. All estimates of error will also include components arising from such points as variation in incubation temperature and errors in counting colonies. The error due to breaking up of clumps contributes to the true sampling error. It may arise between successive dilutions, between replicate plates from the same dilution, or between plates from parallel sets of dilutions.

Apart from possible variations in methods of replication there are differences due to statistical treatment. In Table 30 we have been able to put a number of recent estimates of error of the plate count on a comparable basis. Table 20 and pp. 295–296 should also be consulted.

Table 30. *Error of the plate count*

		No. of samples	Estimated S.D. of a single plate (log)	99% range ($2{\cdot}576\sigma$) %	Estimated C.V. of actual count %
1. Mattick, McClemont & Irwin[1]					
Log of colony no., *plain agar*	1/10 dilution	145	0·135	45–223	31
Two workers, each single tests	1/100 dilution	60	0·170	36–274	
on two subportions of same sample	1/1000 dilution	37	0·129	47–215	
2. Barkworth[39]	Mean log 3·65	31	0·1054	54–186	24
Quintuplicate, milk agar, subportions of same sample	Mean log 4·87	31	0·1105	52–198	25
3. Wilson[2]. Single tests, four workers, subportions of same sample		38	0·1086*	53–190	25†
4. Experiment II					
In duplicate, milk agar:					
(i) Laboratory B. Variability between two plates from *different* sets of dilution tubes		40	0·065	68–147	15
(ii) Laboratory M. Two plates from same dilution		40	0·047	76–132	11
(iii) Laboratory N. Two plates from same dilution		40	0·078	63–159	18
5. Addendum IV					
Laboratory T. Two plates from same dilution		118	0·068	67–150	16
6. Sensitivity experiment					
Quintuplicate, milk agar, from same dilution		169	0·0818	62–163	19

* Estimated from c.v. $\left(\frac{25}{230{\cdot}26}\right)$. † Actual value.

General summary

1. Defining sensitivity as the power of discriminating significantly between milks of different bacterial constitution the plate count is slightly less sensitive than the methylene-blue test observed every 5 min. and slightly more sensitive than the latter observed every half-hour.

2. The logarithm of the plate count is, in our opinion, the correct measure to use. Its coefficient of variation is slightly less than that of the methylene-blue test observed every 5 min.

3. The plate count as formerly used and the methylene-blue test as now used agree in grading in over 80% of cases.

4. Two samples whose reduction times differ by 30 min. are not significantly different. Such a difference is only significant if the result is based on the mean of duplicate tests with 5 min. observations or of triplicate tests with half-hourly observations.

5. Provided transit hazards are eliminated and standard technique is very carefully observed, there is no significant difference in results from different laboratories by either test.

6. Neither test necessarily reflects minor changes or faults in production routine. Under the conditions of our experiment the methylene-blue test was not, in cold weather, a satisfactory indicator of the *numbers* of organisms as distinct from 'activity'.

SUMMARY OF ADDENDA

Addendum I. The distribution, in replicates, of the values of $\log_{10}$ plate count, and of reduction time at both 5 min. and half-hourly observation is such that statistical methods applicable to normal distributions may be used for the examination of such data.

Addendum II. χ^2 values for replicate plate counts are in excess of expectation on the basis of a Poisson distribution. This result is consistent with our other findings and in no way invalidates the comparisons of sensitivity that have been made.

Addendum III. Plain agar(4) gives significantly greater variability between replicates than milk agar.

Addendum IV. Two methods of assessing the final count are considered, and are shown to lead to substantially the same results.

Addendum V. The true error of the plate count is difficult to assess, but estimates by several different workers have been placed on a comparable basis.

GENERAL CONCLUSION

If the same number of replicates is used in both cases the plate count is slightly more 'sensitive', i.e. can distinguish more closely between samples than the methylene-blue test observed every half-hour. Nevertheless, the technique of the methylene-blue test is so much simpler that for routine purposes it is to be preferred, as it is less open to personal and other errors. Further, for routine work the differences in sensitivity are probably of little account. The *sensitivity* of the plate count remains unaffected whether we consider the coefficient of variation to be 12% on the basis of the actual counts or 1·5% on a logarithmic basis.

The authors wish to thank all those who have so generously contributed to the compilation of the data analysed in this paper and who have helped in other ways.

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287. THE FREEZING-POINT OF MILK

I. THE FREEZING-POINT AND SOLIDS-NOT-FAT CONTENT OF THE MILK OF INDIVIDUAL COWS THROUGHOUT A PERIOD OF LACTATION

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(With 2 Figures)

The range and distribution of freezing-point values of the milk of individual cows and of genuine mixed milk from herds have been the object of many investigations (1–10).[1] The results have shown that the freezing-point is the most constant of the physical properties of milk and, for this reason, an eminently suitable indicator of the genuineness of a milk sample. It is known, however, that the freezing-point, though comparatively constant, is subject to minor fluctuations, and a close knowledge of the extent and persistence of such fluctuations is desirable. Only a few data contained in the literature are concerned with samples obtained at consecutive milkings or at periodic intervals, and there appears to have been no systematic investigation of the day-to-day variations or of the extent to which fluctuations may persist over a period of days. Neither is it known whether variations of the freezing-point are in any way correlated with those occurring in the solids-not-fat content. In view of the practice of taking 'appeal to the cow' samples in cases where adulteration is suspected, it is important to know to what extent normal or abnormal characteristics will persist in the milk from a given cow.

Accordingly, we have measured throughout the greater part of a lactation (March–October 1936) the freezing-point and solids-not-fat content of individual samples of milk from three cows.

Experimental

The three cows chosen were:

Peach 10, born August 1932, 1st calf September 1935.

Pippin, born September 1929, 4th calf February 1936.

Flora 16, born October 1929, 4th calf January 1936.

All three were pedigree Dairy Shorthorns, two of them being normal in all respects, whilst the third (Flora 16) was chosen because during the previous lactation she had for short periods given milk of poor chemical composition.

[1] No attempt is made to give a complete list of references; further literature may be found in the papers cited.

Samples were taken at morning and evening milking (about 6.30 a.m. and 3.30 p.m.). As a rule, the freezing-point was determined immediately, but some 5% of the evening samples were kept in cold store overnight before analysis.

At first the Hortvet apparatus was used for the determination of the freezing-point, but this was later replaced by the apparatus described by one of us(11) which makes for greater rapidity of testing. The new apparatus was frequently checked against the Hortvet and the results were found to be in close agreement. All freezing-points are given as depressions (Δ) in degrees centigrade.

The solids-not-fat content was determined as the difference between total solids by evaporation and fat by the Gerber method.

Results

Average values, differences between morning and evening milk

The average values of the freezing-point depressions for Peach 10, Pippin and Flora 16 were 0·546, 0·546 and 0·544° C. for the morning milk and 0·548, 0·548 and 0·546° C. for the evening milk. The corresponding averages for the solids-not-fat were 8·84, 8·64 and 8·19% for the morning milk and 8·91, 8·64 and 8·14% for the evening milk, milk of low solid contents being again produced by Flora 16. The mean values for the freezing-point depressions are in good agreement with those for the milk of many individual cows found by other workers when using the Hortvet technique, e.g. 0·547 (Hortvet(3)), and 0·547 and 0·545° C. (Bailey(4)).

It will be noted that the average freezing-point depression of the morning milk from each cow is 0·002° C. lower than that of the evening milk. Though this difference is very small, in fact equal to the maximum error of the thermometer calibration, it is about four times its own standard error (0·00052° C.) and may, therefore, be accepted as significant. This observation should be clearly distinguished from the contradictory findings reported in the literature which are based on the examination of individual cows over short periods. Our data show that the Δ differences between two consecutive milkings are quite irregular, the Δ of the morning milk being sometimes higher, sometimes lower than that of the corresponding evening milk.

Distribution and extreme range of variation

The percentage distributions of the Δ values for two of the three cows are depicted in Fig. 1. The tendency to lower depressions in the morning milk is quite marked in these graphs. The figures confirm the remarkable constancy of the freezing-point, most values deviating by less than 2% from the average.

The extreme values of Δ and of solids-not-fat content, together with the corresponding solids-not-fat and Δ values, are given in Table 1. No depression lower than 0·529 or higher than 0·560° C. was encountered during the whole of the investigation. Even in the most extreme case (evening milk of Peach 10)

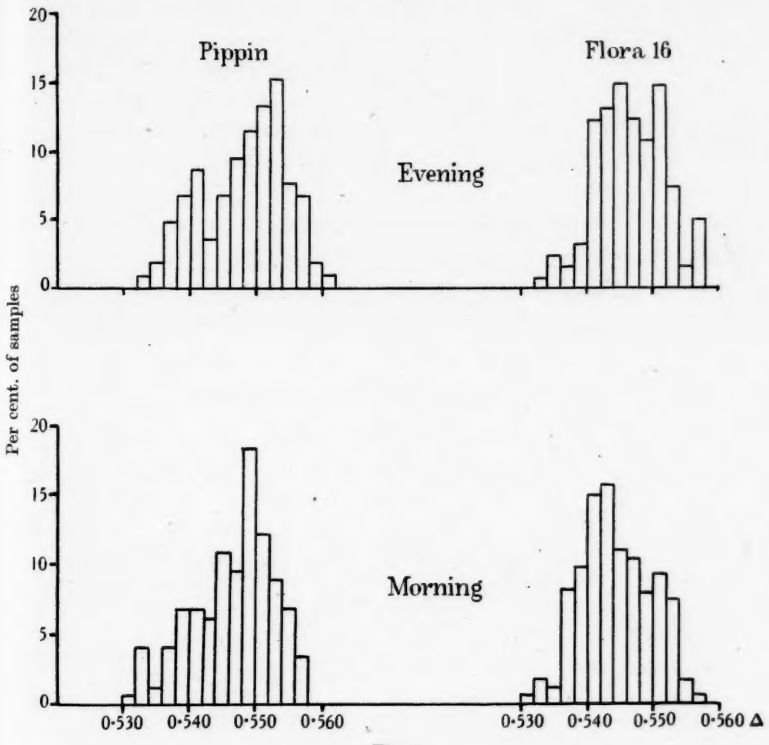

Fig. 1

Table 1. *Extreme range and average values*

		Freezing-point depression				Percentage solids-not-fat			
		Morning		Evening		Morning		Evening	
Cow		Δ	Corresponding solids-not-fat	Δ	Corresponding solids-not-fat	Solids-not-fat	Corresponding Δ	Solids-not-fat	Corresponding Δ
Peach 10	Highest	0·559	8·81	0·559	9·20	10·11	0·550	9·45	0·552
	Lowest	0·532 twice	8·90 8·85	0·529	8·67	8·46	0·543	8·38	0·543
	Average	0·546		0·548		8·84		8·91	
Pippin	Highest	0·557	8·67	0·560	8·37	9·32	0·544	9·25	0·551
	Lowest	0·531	8·06	0·532	8·64	7·45	0·546	7·91	0·540
	Average	0·546		0·548		8·64		8·64	
Flora 16	Highest	0·555 thrice	8·33 8·37 8·42	0·557 twice	8·06 8·03	8·78	0·552	8·81	0·553
	Lowest	0·530	7·82	0·532	7·57	6·97	0·552	7·44	0·547
	Average	0·544		0·546		8·19		8·14	

the freezing-point deviated by less than 3·5% from the mean. None of the extreme values listed in Table 1 occurred at a time when the cows were in season or had been recently served.

Table 1 shows that the solids-not-fat values corresponding to extreme Δ values and the Δ values corresponding to extreme solids-not-fat values are themselves by no means extreme. The data suggest that there is only slight correlation between Δ and solids-not-fat.

Extreme day-to-day variations

Table 2 presents the extreme changes in Δ and solids-not-fat with the corresponding changes in solids-not-fat and Δ observed between one sample and the sample taken at the same time of milking on the following day.

Table 2. *Extreme day-to-day variations.*

(Positive sign denotes an increase)

		Extreme change in freezing-point depression				Extreme change in percentage solids-not-fat			
		Morning		Evening		Morning		Evening	
Cow		Extreme variation Δ	Corresponding variation solids-not-fat	Extreme variation Δ	Corresponding variation solids-not-fat	Extreme variation solids-not-fat	Corresponding variation Δ	Extreme variation solids-not-fat	Corresponding variation Δ
Peach 10	Rising	+0·019	+0·01	+0·022	+0·08	+1·11* (+0·44)	−0·006 (+0·006)	+0·39	—
	Falling	−0·018 twice	+0·03, +0·03	−0·020	−0·04	−1·34* (−0·38)	−0·001 (−0·002)	−0·36	−0·012
Pippin	Rising	+0·015	+0·16	+0·014 twice	−0·37, −0·17	+0·83	−0·013	+0·87	−0·003
	Falling	−0·020	−0·03	−0·016 twice	−0·17, +0·08	−0·71	−0·003	−0·63	+0·002
Flora 16	Rising	+0·017	+0·12	+0·019	+0·23	+0·54	+0·008	+0·96	+0·004
	Falling	−0·020	−0·77	−0·013	—	−0·77	−0·020	−0·44	−0·002

* The extreme variations in solids-not-fat, both rising and falling, recorded for the a.m. milk of Peach 10 arise from one abnormal value (10·11%). For purposes of comparison the next largest variations have been listed in brackets.

With one exception the data support the conclusions drawn from Table 1. The largest sudden variations in either of the characteristics in question are usually accompanied by relatively small changes in the other characteristic, and in nearly one-third of the cases the accompanying change is of the opposite sign. The morning milk of Flora 16 provides the one exception. Here extreme decreases in Δ and solids-not-fat coincide. It is noteworthy that this cow was the one producing poor quality milk.

As in Table 1, none of the extreme variations occurred at the time of coming into season or shortly after service. Thus further doubt is cast upon the statement by Plücker & Steinruck (12) that oestrus causes high freezing-point depressions, a statement already criticized by Bauer (6) on account of the inadequate design of the experiments.

Seasonal and lactational trends

The usual trends in solids-not-fat content associated with the time of year and the state of lactation were observed in all three animals. These trends are too well known to require detailed comment.

The freezing-point depression is generally held to be free from such trends (6, 8, 9, 13), but Buchanan & Lowman (14) have suggested that the Δ values are subject to seasonal changes and are lowest during the time of spring feeding. In Fig. 2 there have been plotted for fortnightly periods the mean (×) and maximum (○) Δ values for two of the animals. Unfortunately, we were unable to obtain the curves for the third animal (Peach 10), as some of the records relating to this cow had been lost at the time of writing. How-

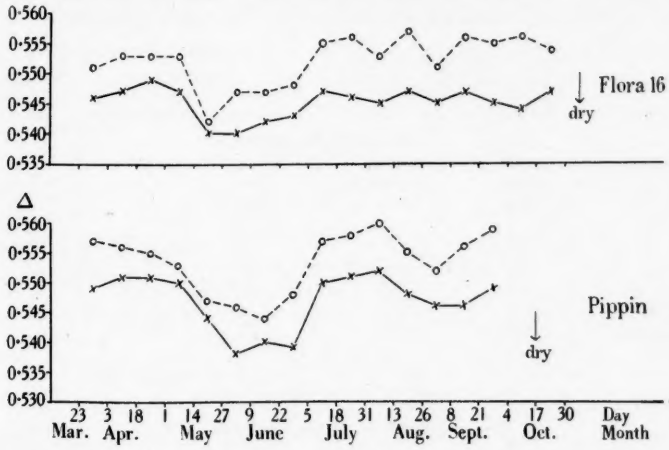

Fig. 2. Fortnightly periods: ×——× = mean Δ; ○- - -○ = maximum Δ.

ever, Fig. 2 shows that both the average values and the maximum values exhibit a slight but definite minimum during the period from 14 May to 5 July. It is noteworthy that during this period not a single depression of 0·550 or more was recorded, whereas such values occurred with regularity throughout the remainder of the experiment. As the two cows were in the same state of lactation, it cannot be decided from the data whether the low freezing-point depressions were due to the state of lactation or to a seasonal effect. It can, however, be stated that further evidence on this point has meantime been collected and will be published shortly, confirming the view of Buchanan & Lowman that the phenomenon is seasonal and closely bound up with the turning out of the animals to fresh young grass.

Mean fluctuations

In view of the above-mentioned trends the standard deviation from the fortnightly average has been chosen as a measure of the mean fluctuation. In this way the effect of major trends is eliminated, whilst the effect of any

possible persistence of fluctuations for a few days remains included. The standard deviations are given in Table 3, both as absolute values and as coefficients of variation. Again the data serve to emphasize the high degree of constancy of the freezing-point. In no case is the coefficient of variation of Δ as great as 1%, whilst that of solids-not-fat ranges from 1·4 to 3·0% with an average of 2·4%. The magnitude of the Δ fluctuations does not differ systematically as between morning and evening milk, but the coefficient of variation of the solids-not-fat content of evening milk is less than that of the morning milk in the ratio of about 5 to 6. This difference is statistically significant ($P = 0{\cdot}020$). Evening milk is thus more uniform in solids-not-fat than morning milk.

Table 3. *Standard deviations from fortnightly averages*

Cow	Standard deviation		Coefficient of variation		Difference between last 2 columns (M. – E.)
	Morning	Evening	Morning	Evening	
		Freezing-point depression			
Peach 10	0·0049	0·0048	0·90	0·88	+0·02
Pippin	0·0048	0·0050	0·88	0·91	−0·03
Flora 16	0·0045	0·0050	0·83	0·91	−0·08
				Mean	−0·03
				Standard error	0·03
		Percentage of solids-not-fat			
Peach 10	0·177	0·126	2·00	1·42	+0·58
Pippin	0·238	0·209	2·78	2·42	+0·36
Flora 16	0·247	0·213	3·02	2·62	+0·40
				Mean	+0·45
				Standard error	0·068

Persistence of fluctuations

The persistence of fluctuations was assessed by correlating the standard deviation of the difference between two tests with the standard deviation from the mean for the following four intervals: between successive mornings, between successive evenings, between morning and evening of the same day, and between the evening of one day and the morning of the next day. The calculation showed that fluctuations in Δ are not persistent and decrease to about one-fifth of their value in one day. Fluctuations in the solids-not-fat content, on the other hand, persist for several days, but the statistical technique applied was not suitable to assign an exact period.

Summary

The results of regular determinations of the freezing-point and solids-not-fat content of the milk of three Shorthorn cows over a period of more than six months are reported.

The known constancy of the freezing-point depression was confirmed, the Δ values deviating generally by less than 2% and, in the most extreme case, by no more than 3·5% from the mean of $0{\cdot}546 \pm 0{\cdot}002°$ C. No evidence was

found of any influence of the state of lactation on the freezing-point depression, but a slight but definite decrease persisting for some weeks occurred at the time of year at which ample supplies of spring pasture became available to the animals.

For each animal the freezing-point depression of the morning milk was on the average 0·002° C. lower than that of the evening milk. Correlation between daily variations in Δ and solids-not-fat was low. Extreme values or variations of either of these quantities coincided only rarely with those of the other.

Extreme values or variations were not associated with periods of coming into season or with service.

Fluctuations of the freezing-point were not persistent, whilst those of the solids-not-fat content tended to persist for several days.

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288. A SIMPLIFICATION OF THE SCOTT BLAIR-COPPEN TEST FOR THE PITCHING CONSISTENCY OF CHEESE CURD

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(With 1 Figure)

Introduction

Scott Blair & Coppen (1) have described a test for the consistency of firm cheese curd during the process of scalding and at the pitching point. This test gives the cheese maker an objective physical measure of a property which he is used to assessing subjectively by handling the curd, and should ensure a more constant condition in the curd from vat to vat and from day to day.

In the test as originally described, a small cylindrical wire-gauze basket is filled with curd from the vat, inverted 5 sec. later on to a gauze disk so as to make a flat top surface, reinverted after 15 sec.,[1] weighed after 27 sec., then turned out after 35 sec. on to a flat surface, the height of the moulded cylinder of curd being measured after 50 sec.[2] The ratio of the weight of curd (W g.) to the height of the moulded cylinder (h cm.) is, at least in the neighbourhood of the pitching point, a measure of the superficial density of the curd, and falls as the scalding process proceeds owing to the progressive replacement of whey by air. In the firmer curd, the particles retain their shape when the curd is cast into the form of a cylinder, and the whey drains out readily, being replaced by air.

Since the drainage process is proceeding during the weighing and height measurements, the values of W and h diminish progressively with time, and the arbitrarily chosen time intervals given above were always carefully followed. In some factories in which the test was in use, this 'timing' was regarded as a disadvantageous feature of the test, partly because in a big factory where the instrument may be placed at some distance from the vat there is hardly time to carry the sample to the instrument in the period allowed, and still more because some people find difficulty in carrying out the necessarily rather rapid manipulations and observing a stop-watch at the same time.

We therefore felt that it would be advisable to ascertain (*a*) whether the test could be carried out at a slower rate and whether, if all the times were to be increased in approximately the same proportions, the values of W/h

[1] All times are taken from the moment when the samples are removed from the vat.

[2] For details see original papers.

would be appreciably affected, and (*b*) how serious are the errors in W/h when known differences are deliberately made in the timing.

Experimental

In the Cheddar-cheese factory in which the tests were done, there are six vats of approximately 750 gal. capacity, the first two or three of which (renumbered 7, 8, 9[1]) are used again later in the day, thus giving a total capacity of about 6000 gal. Some of the milk was pasteurized and some was raw or mixed, but no significant effect on W/h values due to pasteurization was observed, and this factor is not discussed in the present paper. Unless the acidity of the whey was markedly abnormal the vats were pitched 'by the clock' so that subjective judgements of curd firmness were seldom made. Tests were made by us on almost all the vats during the course of a week's manufacture, and in some cases samples were taken frequently during the whole of the scalding process. Temperatures were recorded throughout and acidities at cut and pitch were noted. No significant correlation was found between W/h at pitch (P), and either maximum temperature of scald, rate of scald, or acidity, although, since constancy of these factors was sought by the cheese maker, the variations found in them were naturally not extreme. Taking the mean value of P for each day, we found that this figure tended to be higher (curd softer) when the acidity of the starter was high, thus confirming our growing conviction that the nature of the starter plays an extremely important part in defining the firmness of the curd. The data bearing on this point were insufficient to merit statistical analysis.

The mean P values and temperatures of scald (θ) for each vat were worked out for the whole week and are given in Table 1.

Table 1

Vat	1	2	3	4	5	6	7	8
Mean P	18·41	18·11	18·73	18·32	18·42	17·66	18·17	18·26
Mean θ (° F.)	105·5	104·5	105	103·5	105	105	105	103

Vat 6 was nearest the tipping tank and was filled most quickly. Vat 3 was the slowest filled. The cheese maker had previously observed that vat 6 tended to give a tough and vat 3 a rather soft curd. This was confirmed by the figures in Table 1. It is possible that the greater firmness of curd in vat 6 was also influenced by draughts from a frequently opened door, but it is clear from Table 1 that the effects were not due to differences in temperature of scald. The test is thus shown to serve as a useful check on observations such as the above.

A histogram of P values for all vats is given in Fig. 1. The mean is 18·2, the mode about 18·6 and the P.E. 0·08.

It is suggested that such histograms, if drawn up for a week's production from time to time, should prove very useful to the cheese maker as an indi-

[1] Only occasionally used.

cation of (*a*) the average pitching consistency of his curd by comparison with previous occasions, and (*b*) the range over which this consistency is varying. Both these factors will be influenced by changes in the milk supply and in the manufacturing process and should be quantitatively followed.

The 'spread' of the curve is, naturally, greater in factories where curd is 'pitched' by the clock than in those where each curd is tested by the cheese maker before deciding to pitch. The use of the pitching test as a criterion would naturally make for the highest consistency of all.

In the experiments now to be described tests were carried out by both of us, but no significant differences were observed which could be attributed to the personal factor.

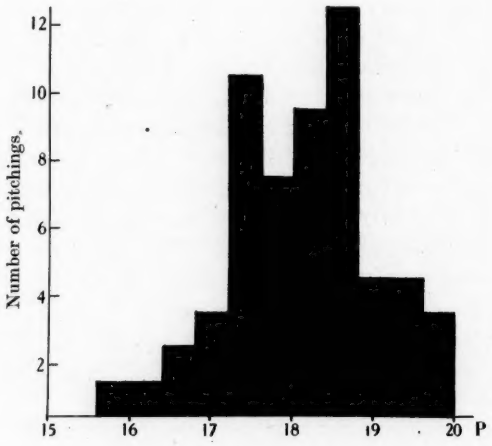

Fig. 1

Experiment 1. To study the effect of approximately doubling all the times (except that of the first inversion, which was always kept at 5 sec.) on the values of W/h.

The following times were used:

Original method (A): 5, 15, 27, 35, 50 sec.

Slower method (B): 5, 30, 45, 60, 90 sec.

The results are given in Table 2 and show clearly that the slower process has made no appreciable difference in the values of W/h. This was confirmed by other experiments made by Dr F. M. V. Coppen at the British Dairy Institute, Reading.

Experiment 2. The above experiment was repeated, but a third rate (*C*), too slow to be convenient in ordinary practice, was also included. The times for *C* were 5, 45, 60, 90, 135 sec. The results, given in Table 3, confirm the earlier experiment and extend its validity to still slower tests.

Table 2*

Vat 1. Acid at pitch 0·18

t	80	77	61	58	56	53	51	46	41	35	30	18	11	10	7	1	0
Temp.	85	85	—	88	90	92	94	96	98	104	102	106	106	106	—	105	105
Method	B	A	B	B	A	B	A	A	B	B	A	B	A	B	B	A	B
W/h	30·4	29·1	27·9	26·2	26·2	26·2	25·0	25·3	24·3	22·0	22·6	22·2	20·4	20·2	19·8	20·0	19·7

Vat 2. Acid at pitch 0·18

t	63	49	45	44	39	37	29	24	15	11	7	2	0
Temp.	90	97	98	98	100	—	102	102	102	103	102	103	103
Method	A	A	B	B	A	B	A	A	B	B	B	A	B
W/h	>24·1	24·0	24·0	23·5	22·1	22·2	21·0	19·9	19·7	21·4?	18·4	18·4	18·5

Vat 3. Acid at pitch 0·195

t	63	59	56	50	48	45	31	30	27	14	11	9	3	0
Temp.	88	89·5	90	93	94	96	106	107	108	108	107	107	107	107
Method	A	B	B	A	B	B	A	B	B	B	A	B	A	B
W/h	26·2	25·6	25·3	24·8	24·8	24·4	23·1	23·1	22·6†	19·2	18·9	18·9	18·9	19·1

Vat 4. Acid at pitch 0·18

t	64	62	58	47	45	42	29	20	17	14	2	0
Temp.	87	88	90·5	98	99	101	107	105	105	104	104	104
Method	A	B	B	A	B	B	A	B	A	B	A	B
W/h	27·2	26·4	27·5	24·4	24·3	23·8	21·4	21·2	21·2	21·2	19·5‡	19·6

Vat 5. Acid at pitch 0·17

t	58	56	52	31	29	23	13	10	2	0
Temp.	96	98	97	107	107	107	106·5	106	105·5	105
Method	A	B	B	A	B	B	A	B	A	B
W/h	25·5	25·9	24·6	21·2	21·5	20·3	19·9	20·0	18·7	18·6

Vat 6. Acid at pitch 0·19

t	61	59	48	47	17	9	4	0
Temp.	90	91	97	—	106	105	105	105
Method	A	B	A	B	B	B	B	B
W/h	25·7	25·2	23·8	24·3	19·2	18·4	18·4	17·3

Vat 7. Acid at pitch 0·195

t	47	26	23	18	6	3	0
Temp.	97	108	108	107·5	108	108	108
Method	B	A	B	B	B	A	B
W/h	24·2	22·2	21·3	33·0?	19·2	19·7	19·7

Vat 8. Acid at pitch 0·18

t	46	42	39	26	24	21	12	8	5	2	0
Temp.	97	97	98	105	106	106	105	105	105	104	104
Method	A	B	B	B	A	B	A	B	B	A	B
W/h	24·0	23·3	23·7	22·7	21·7	21·0	20·5	19·3	19·5	19·2	18·6

* In this and all subsequent tables *t* is the time (min.) before pitching.

† Lumpy curd. ‡ Vat not adequately stirred.

Table 3

Vat 1. Acid at pitch 0·16

t	60	58	55	51	45	43	39	31	28	25	13	8	2	0
Temp.	91·5	91·5	93	94	97	98	100	105	107	107	105	104·5	104·5	103·5
Method	A	B	A	C	A	B	C	B	C	A	A	B	C	A
W/h	27·1	26·0	26·3	25·1	24·9	24·9	24·6	23·8	22·8	22·3	20·6	19·7	20·0	19·6

Vat 2. Acid at pitch 0·16

t	58	55	52	48	31	28	22	9	5	2	0
Temp.	93	93·5	95·5	98	107	108	108	106	105·5	105	105
Method	A	B	C	C	B	A	C	C	B	C	A
W/h	24·8	25·0	28·3?	24·2	22·1	21·3	20·0	19·5	18·5	18·3	19·1

Vat 3. Acid at pitch 0·19

t	68	63	62	54	50	48	38	35	22	17	14	11	5	3	0
Temp.	88	89	90	97	100·5	102	106	108	108	106·5	106·5	106·5	106	105·5	105·5
Method	A	C	B	C	B	A	B	A	C	B	A	C	A	B	A
W/h	28·6	27·7	27·2	25·4	25·8	24·6	22·8	22·5	22·1	20·3	20·4	20·3	19·9	18·7	18·9

Vat 4. Acid at pitch 0·185

t	58	55	48	25	22	18	7	4	0
Temp.	96·5	101	102	106	106	105	104	103	103·5
Method	B	C	A	B	C	A	C	B	A
W/h	24·4	24·5	22·8	19·5	19·6	19·0	18·5	17·2	18·3

Table 3 (*continued*)

Vat 5. Acid at pitch 0·19

	60	57	55	13	7	2	0		*t*	25	11	8	4	0
Temp.	93	94·5	96	107·5	107	107	107		Temp.	105	103·5	103·5	103·5	103
Method	C	A	B	A	C	B	A		Method	A	B	C	B	A
W/h	25·5	24·7	24·1	18·7	18·0	18·7	17·5		*W/h*	20·4	18·6	19·5	18·7	17·9

Vat 7. Acid at pitch 0·195

	31	28	25	3	0		*t*	44	42	40	27	24	22	14	0
Temp.	104*	105*	105*	104*	104·5*		Temp.	100	100·5	101	103·5	104	103·5	102	102
Method	A	B	C	B	A		Method	A	B	C	B	A	C	A	A
W/h	19·6	18·7	18·8	17·3	16·3		*W/h*	23·2	24·1	23·3	22·6	21·5	21·3	20·5	19·5

Vat 8. Acid at pitch 0·195

Vat 9. Acid at pitch 0·18

t	45	43	39	27	25	22	19	7	4	0
Temp.	96	98	101	109*	109*	109·5*	109	107	106·5	107
Method	B	A	C	A	B	B	C	C	B	A
W/h	28·5	25·0	24·1	21·6	19·8	19·2	19·5	18·8	17·5	18·0

* Lumpy curd.

Experiment 3. To study the effect of 10 sec. variations either way in (*a*) the time between the first and second inversions and (*b*) the time between the last inversion and the measurement of *h*.

The timings used were as follows:

	h_1	h_2	h_3
A:	5, 30, 45, 60,	80,	90, 100.
B:	5, 20, 35, 50, 70,	80,	90.
C:	5, 40, 55, 70, 90, 100, 110.		

The data are given in Table 4.

Table 4

Vat 1. Acid at pitch 0·16

t	54	51	47	39	37	33	22?	9	5	0
Temp.	92	93·5	96	100·5	102	104	107·5	107	106	106
Method	A	B	C	C	A	B	B	C	B	A
W/h_1	25·1	25·2	23·6	23·8	23·6	23·9	—	20·0	19·7	19·5
W/h_2	25·7	25·6	24·2	24·0	23·8	24·1	22·6	20·2	20·0	19·6
W/h_3	26·2	26·0	24·8	24·4	24·2	24·6	—	20·2	20·1	19·7

Vat 2. Acid at pitch 0·16

t	30	25	23	16	13	10	6	3	0
Temp.	107	106	106	105	105	105	104·5	104	104
Method	A	B	C	C	A	B	C	B	A
W/h_1	21·2	20·6	20·1	19·5	20·2	19·9	18·9	19·8	18·2
W/h_2	21·6	20·9	20·3	19·8	20·2	20·2	18·9	19·9	18·4
W/h_3	21·8	21·2	20·6	20·0	20·3	20·4	19·2	20·5	18·5

Vat 3. Acid at pitch 0·17

t	30	25	20	15	9	5	3	0
Temp.	106	107	107	107	107	106·5	106	106
Method	A	A	B	C	A	B	C	A
W/h_1	—	—	20·2	19·2	18·4	18·8	17·9	18·6
W/h_2	21·8	21·4	20·4	19·4	18·7	19·1	18·1	18·6
W/h_3	—	—	20·6	19·6	18·9	19·4	18·4	19·0

Vat 4. Acid at pitch 0·17

t	17	14	11	5	2	0
Temp.	105	104	104	103·5	103·5	103
Method	A	B	C	B	C	A
W/h_1	18·4	18·9	18·9	17·9	17·6	17·4
W/h_2	18·6	19·4	19·0	18·0	17·8	17·5
W/h_3	18·8	19·4	19·4	18·4	18·0	17·6

Vat 5. Acid at pitch 0·17

t	8	0	
Temp.	106	106	
Method	B	B	
W/h_1	18·5	18·5	
W/h_2	18·8	18·8	
W/h_3	19·1	19·0	

Vat 6. Acid at pitch 0·175

	21	17	13	7	5	0
Temp.	107	106·5	106·5	106	106	105·5
Method	A	B	C	B	C	A
W/h_1	19·3	19·5	19·5	18·4	17·9	18·3
W/h_2	19·6	19·9	19·6	18·8	18·2	18·8
W/h_3	19·9	20·1	19·7	18·9	18·4	18·9

Table 4 (*continued*)

Vat 7. Acid at pitch 0·18

t	50	47	43	33	22	18	8	4	0
Temp.	94	95	96	101·5	108	108	106	106	106
Method	A	B	C	C	B	A	B	C	A
W/h_1	24·0	24·3	23·5	22·2	22·2	20·7	21·0	17·9	18·6
W/h_2	24·7	24·7	23·6	22·6	22·6	20·8	21·2	18·0	19·0
W/h_3	25·2	25·0	24·3	22·8	22·8	21·1	21·5	18·4	19·1

Vat 8. Acid at pitch 0·18

t	25	23	19	13	11	5	0
Temp.	106	106	105·3	106	105	106	106
Method	A	B	C	A	C	B	A
W/h_1	25·5	19·5	19·5	18·4	17·8	18·0	17·7
W/h_2	25·5	20·0	19·6	18·5	18·1	18·4	18·1
W/h_3	25·6	20·1	19·7	18·8	18·2	18·6	18·2

We conclude that when the curd is approaching the pitching consistency, even such large errors as 20 sec. in the timing of the critical stages of the test will make surprisingly small differences in the values of W/h. In the earlier stages of scald, when whey is escaping more slowly from the mass of curd, the discrepancies produced are greater, but it is safe to say that, for practical purposes, errors of several seconds may be made without detracting from the value of the test.

This suggested that the test might be carried out without the use of a watch, the times (series B in Exp. 1) being estimated. The first inversion was timed by slowly counting five, but the rest of each test was performed by trying to keep the correct rhythm without counting. This constituted Exp. 4.

The results given in Table 5 would doubtless be improved with practice but, as a first attempt, the results show clearly that the test can be carried out with adequate accuracy without a watch and conveniently at about half the speed proposed in the earlier papers, though care must be taken not to deviate too widely from the correct timing.

Table 5*

Vat 1. Acid at pitch 0·19

t	67	62	53	50	45	41	32	28	8	6	0
Temp.	88	92	96	96·5	99·5	101	105	106	105·5	105	105·5
Method	T	T	T	U	U	T	T	U	T	U	T
W/h	27·6	26·2	25·3	24·4	22·2	23·2	21·4	21·3	18·6	18·1	17·4

Vat 2. Acid at pitch 0·195

t	59	56	45	43	25	19	14	11	6	2	0
Temp.	91	92·5	98	100	106	106	105·5	105·5	105	104·5	104·5
Method	T	U	T	U	T	U	T	U	T	U	T
W/h	27·7	24·7	27·0	23·1	19·8	19·5	18·9	19·0	18·7	18·4	18·5

Vat 3. Acid at pitch 0·19

t	51	48	37	34	27	25	15	13	3	0
Temp.	97	98·5	104	105	105	106	105	104·5	104	104
Method	T	U	T	U	T	U	T	U	U	T
W/h	25·2	25·5	23·0	22·2	21·5	21·0	20·6	19·9	18·4	19·4

Vat 4. Acid at pitch 0·19

t	54	51	37	35	22	20	12	10	5	0
Temp.	98·5	99·5	105	105·5	105·5	105	104·5	104·5	104·5	104·5
Method	T	U	T	U	T	U	T	U	U	T
W/h	23·3	22·9	21·2	20·5	18·6	18·8	17·7	17·9	17·8	17·9

* T = timed, U = untimed.

Table 5 (*continued*)

Vat 6. Acid at pitch 0·19

t	26	23	17	13	4	0
Temp.	105	105	105·5	105	105	105
Method	T	U	T	U	U	T
W/h	21·8	21·0	20·2	19·9	18·0	18·0

Vat 7. Acid at pitch 0·19

t	59	55	44	41	28	24	10	7	0
Temp.	90	91·5	98	100·5	102	102	105	105	105
Method	T	U	T	U	U	T	T	U	T
W/h	24·8	24·6	22·9	22·3	20·2	19·9	18·1	17·8	17·7

Vat 8. Acid at pitch 0·19

t	39	36	32	23	16	13	9	5	2	0
Temp.	97	99	103	105*	104	104	103·5	103·5	103	103
Method	T	U	T	T	U	T	U	T	U	T
W/h	25·3	26·8	23·2	19·1	18·5	18·6	18·2	18·7	17·7	17·4

* Lumpy curd.

Conclusions

1. The Scott Blair-Coppen pitching test has been intensively studied during a week of commercial Cheddar-cheese making. It is shown that the consistency (P) of the curd at pitching (which was done 'by the clock') was not correlated with the variations in temperature, rate of scald or acidity which occurred in the normal course of manufacture. The starter acidity and conditions of adding the starter (rate of filling vats, etc.) appear to influence P.

2. A histogram of P values for a week's production is given. Such histograms should be of value to cheese makers, if made from time to time, as an indication of variability of manufacture especially under changing war-time conditions.

3. The test, previously carried out in 50 sec., each process being carefully timed to the nearest second, can be as effectively done more slowly if approximately the same proportional times are used for each stage. In some large factories and in the hands of less skilled personnel, the slower test is an advantage.

4. Much less accuracy of timing is required in order to get satisfactory results than was previously supposed necessary. The test may even be done without the use of a watch if care is taken not to deviate too widely from the specified times.

The relationship between the values of P obtained for each cheese and its 'body' when ripened will, it is hoped, form the subject of a later communication.

We wish to thank the Milk Marketing Board for permission to carry out this work at their Sturminster factory and Mr C. Lewis, Miss M. L. Simpson and the other members of the staff for the help and co-operation which we received throughout the experiments.

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289. THE RHEOLOGY OF BUTTER

I. METHODS OF MEASURING THE HARDNESS OF BUTTER

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(With 4 Figures)

The physical properties of butter as they appear to the grader are summed up in the term 'body'. In determining the score to be allotted for 'body' the grader takes into account a number of properties which include hardness, crumbliness, greasiness and stickiness. Of these properties hardness, which may be defined as resistance to shear, is the most important and the most readily measured. Methods for the quantitative determination of hardness have been used by many of the numerous workers who have interested themselves in the consistency and spreading capacity of butter. The methods, with one or two exceptions, have measured the hardness in arbitrary units under a set of conditions particular to each method. The manner of expressing the results has often shown a lack of appreciation of the principles involved in the determination, while deductions as to the spreading capacity of butter have frequently been made from measurements carried out under conditions differing considerably from those applying when butter is spread with a knife.

The methods of measurement which have been used may be classified as follows:.

(1) *Compression methods.* A cube or cylinder of butter is compressed between parallel plates by the action of weights. Two of the three variables, load, time and compression, are kept constant and the third used to measure the hardness (e.g. Perkins(1), Hunziker, Mills & Spitzer(2), Lyons(3), Coulter & Combs(4)). Scott Blair(5) has described an apparatus of this type and has shown how the results may be expressed in absolute units.

(2) *Penetrometer methods.* A rod, usually fitted at the lower end with a ball or cylinder of larger diameter than the rod, is pressed into the butter by means of weights. One of the three variables, depth of penetration, load and time, is measured, the other two being kept constant. Lyons(6) used a modified penetrometer method in which a conical weight was dropped from a given height on to the test piece of butter, the hardness being expressed as the volume of butter displaced. Kruisheer & den Herder(7) have described two types of penetrometer for use with butter packed in tubs or boxes. In the first type the penetrometer rod was loaded with weights, and the penetration after a given time was measured. In the second method the plunger was

forced into the butter at a given rate either by hand or by an electric motor, and the resistance was measured by a spring balance.

(3) *Extrusion pressure.* Griffiths (8) has described an apparatus in which a cylinder closed by a plate with a sharp-edged orifice is filled with butter and the minimum pressure to produce extrusion is measured. A modification of this apparatus was used by Sargent (9).

(4) *Resistance to cutting by a wire* (Sectility). Coulter & Combs (4) used an apparatus in which a 1 lb. pat of butter was cut by a number of wires stretched on a frame from which a 50 lb. weight was hung. Hardness was expressed as time of cutting.

(5) *Sagging beam method:* This method, developed by Leighton, Leviton & Williams (10) for use with ice cream, has been applied by Coulter & Combs (4) to butter. A cylindrical beam of butter is supported by its ends in a horizontal position, and the rate of sag is determined. The method is applicable only at temperatures at which the butter is soft.

Up to the present no attempt has been made to compare the results obtained by the various methods, nor has it been shown that they are directly related to the ease with which butter may be spread on bread. One apparatus (de Lacy (11)) has been devised in which an actual spreading action is used. A sample of butter is spread on a sheet of cork by means of an inclined knife fixed to the lower side of a trolley which is drawn forward by weights attached to a cord passing over a pulley. In addition to various mechanical faults which introduce friction this apparatus has no provision for temperature control, and no means for measuring rate of spreading.

Experimental

During one season, measurements were made on a number of butter samples with the Scott Blair apparatus, modified to permit accurate temperature control. A bath 8 in. square and 3 in. deep was fixed to the baseboard of the apparatus. Through this bath water from a thermostat was circulated. During the measurement the sample was completely immersed in the water. A correction was made for loss of weight due to the immersion of the lower portion of the loading column in the water.

It soon became evident that although the instrument gave an approximate measure of hardness, the results were not sufficiently reproducible to permit the detection of small differences in hardness, or to make possible a study of the rheological properties of butter. The chief difficulty encountered was a crumbling of the test piece on compression, a difficulty already mentioned by Scott Blair. Closer observation showed that butter normally has a laminated structure, presumably produced in the working process in the same way as the layers in flaky pastry are formed when the pastry is rolled out. When a cylinder of butter is compressed there occurs a splitting along the planes of cleavage rather than an actual flow of the butter. Under these conditions the assumptions made in calculating the results in absolute units

are no longer valid. Reproducibility is poor, since it is a matter of chance in which direction the planes of cleavage in a particular test piece will run. If they are horizontal there will be little splitting, while if they are vertical or sloping, splitting will occur. Friction in the bearing of the loading column was found to be an additional source of error. Some test pieces spread eccentrically, thus producing a side thrust on the bearing and greatly increasing the friction.

A search was made for a more reproducible method of measuring butter hardness. It was evident that any method depending on the compression of a cube or cylinder of butter would be subject to difficulties through crumbling or splitting of the sample. Trials showed that the application of penetrometer methods to a block of butter which was not supported on the sides also caused splitting. Even where the sample was placed in a closely fitting container there was some flaking off of the butter round the point of penetration. Trials with the extrusion pressure apparatus used by Sargent showed that while it was more promising, there were practical difficulties in accurately maintaining and measuring the pressure during a determination. Furthermore, it would have been necessary to provide a means of measuring the rate of extrusion by determining either the weight or volume of butter extruded in a given time.

A trial of the sectility method showed that it could give satisfactorily reproducible results and had other desirable features. As with the penetrometer method it was necessary to support the block of butter on the sides to prevent splitting, but when this was done the loaded wire sank at a constant rate through the butter. The sectility method appears to have been little used in rheological work. The apparatus employed by Coulter & Combs(4) was suitable only for obtaining an empirical measure of hardness. A more refined apparatus which was used for determining the hardness of soap has been described by Bowen & Thomas(12). A bar of soap mounted on a trolley was moved forward at a constant speed by an electric motor. As it moved the soap was cut by a wire attached to a second trolley. The load on the wire was recorded on a revolving drum by a spring balance.

For the present investigation a simpler apparatus which permits the measurement of resistance to cutting at various rates has been devised. A drawing of the apparatus is given in Fig. 1. A steel wire (no. 22 s.w.g. diam. 0·71 mm.) was stretched across the arms of a V-shaped piece cut from 18 gauge stainless steel sheet. This in turn was attached to a wire frame resembling a balance pan. To the top of the frame a scale graduated in millimetres was fixed. The butter was held in a frame made from a strip of 18 gauge stainless steel sheet 2 cm. wide which was bent to form three sides of a rectangle. The fourth side was formed by a bridge piece of the same strip which fitted over the ends of the first piece. The frame and supporting clip are shown in isometric projection on the right-hand side of Fig. 1. This frame was pressed into the sample and the projecting parts of the butter were

trimmed off with a wire. The block of butter thus formed measured approximately 6 by 3 cm., while the thickness between the exposed surfaces was exactly 2 cm. The bridge piece was removed, and the frame containing the butter was placed in a support on the bottom of a small water-bath through which water was circulated from a thermostat. The cutting wire was placed on top of the block of butter so as to be at right angles to the exposed faces and the bridge piece was replaced. Weights were placed on the pan and the time for each 5 mm. fall was determined by observation of the movement of the scale past a cross-wire held on the stand which supported the water-bath. Where very slow rates were to be measured the scale was observed through

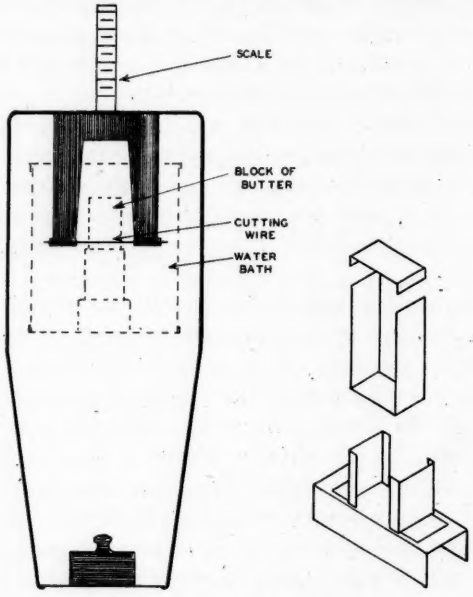

Fig. 1. Diagram of sectility apparatus.

a low-power microscope fitted with a cross-wire. In recording the load the weight of the pan and cutting wire (78 g.) was added to that of the weights used. Since there were no guides or bearings for the moving part of the apparatus frictional errors were eliminated. The centre of gravity of the system was sufficiently far below the cutting wire for the wire to remain horizontal throughout the determination.

Provided the sample was uniform and free from holes or cracks, the time of cutting of each 5 mm. depth of the sample was practically the same. A shorter time for one section than for the others could usually be accounted for by the presence of a hole in that portion of the test piece, and the result for that section was therefore discarded. The mean of the times for the remaining 5 mm. sections was used to calculate the rate of cutting for the

sample. Determinations were usually made with at least four different loads giving rates of cutting varying from 10^{-3} to 10^{-1} cm./sec.

Effect of diameter and length of wire

Some experiments were made to determine the effect of diameter of cutting wire used and of length of wire in contact with the butter. For the second section of the experiment blocks of butter of thickness from 5 mm. up to 2 cm. were used. The results are shown graphically in Fig. 2 (curve 1) and Fig. 3 (curve 1). In each case the load recorded is that required to produce a rate of cutting of 10^{-2} cm./sec. Since the curves are almost linear, the load required to produce a given rate of cutting may be taken as approximately

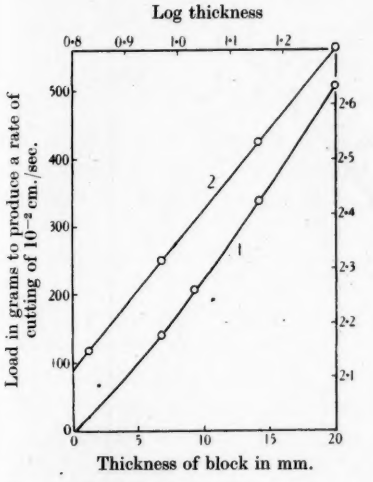

Fig. 2. Effect of thickness of block of butter on load required to produce a given rate of cutting by a wire.

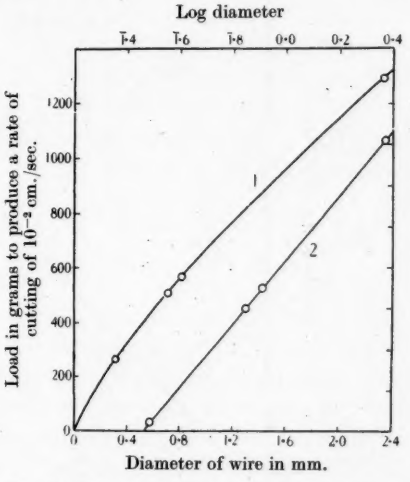

Fig. 3. Effect of diameter of wire on load required to produce a given rate of cutting.

proportional to the diameter of the wire and the length in contact with the butter, where the diameters of the wires or thicknesses of the blocks of butter compared do not differ greatly. A more accurate relationship is obtained from the graphs of $(\log d)/(\log W)$ and $(\log l)/(\log W)$ (Fig. 3, curve 2 and Fig. 2, curve 2), which are accurately linear over their whole length. From these it may be shown that the relation is

$$W \propto d^{0.8} \times l^{1.2}.$$

It seems safe to assume that the load required to produce a given rate of cutting will be directly proportional to the viscosity at that particular rate. (It will be shown later that viscosity varies with rate of cutting.) The results obtained may therefore be treated as viscosities although the units used are arbitrary. This would not be the case if time of cutting with a given load

were used, as was done by Coulter & Combs, or if depth of penetration with a penetrometer under a given load were measured.

Comparison with Scott Blair apparatus

A comparison was made of the results obtained by the sectility method and by the Scott Blair method on some fifty samples. The results are shown graphically in Fig. 4, in which the load in grams required to produce a cutting

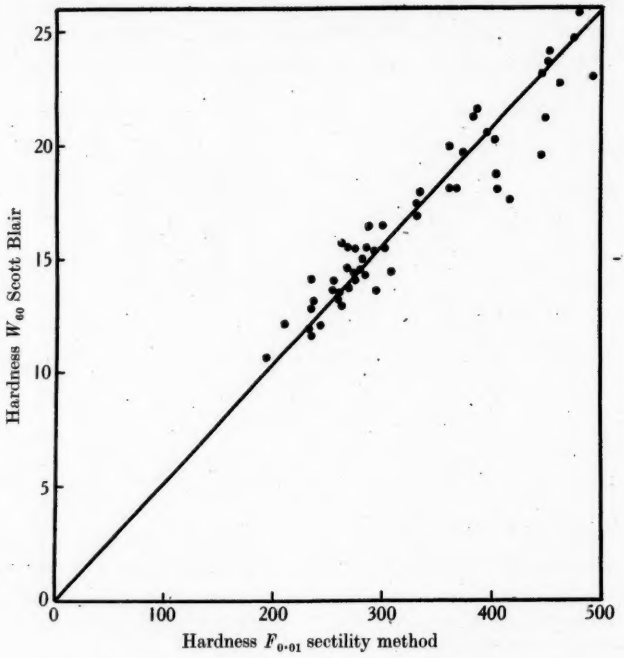

Fig. 4. Comparison of results obtained for hardness of butters by Scott Blair and by sectility methods.

rate of 10^{-2} cm./sec. is plotted against the load in pounds with the Scott Blair apparatus required to compress a cylinder of butter 2 cm. high to a height of 1·21 cm. in 60 sec. Average rates of spreading were thus similar in the two cases (0·010 cm./sec. and 0·013 cm./sec.). The points all lie close to a straight line passing through the origin, and thus show a direct proportionality between the results by the two methods. It is evident, therefore, that both measure the same property of the butter.

Using the data of Fig. 4 the average relationship between the results by the two methods is found to be

$$W_{60} = 0{\cdot}516 F_{0{\cdot}01},$$

where W_{60} = load in pounds with the Scott Blair apparatus to give a compression time of 60 sec., and $F_{0\cdot01}$ = load in grams with the sectility apparatus to produce a rate of cutting of 0·01 cm./sec.

The results obtained by the sectility method may be converted to absolute units as follows:

It has been shown by Scott Blair that with his apparatus

$$\eta = 0\cdot01896 Wt \text{ megapoises.}$$

Where $t = 60$ sec., $\eta = 1\cdot1376 W_{60}$ megapoises;

substituting $F_{0\cdot01}$ for W_{60} we have

$$\eta = 1\cdot1376 \times 0\cdot0516 F_{0\cdot01}$$
$$= 0\cdot0586 F_{0\cdot01} \text{ megapoises.}$$

If the rate of cutting is V cm./sec. and the load F_v

$$\eta = 0\cdot0586 F_v \times 0\cdot01/V$$
$$= 0\cdot000586 F_v/V \text{ megapoises.}$$

On account of the crumbling of samples when tested by the Scott Blair method the values will probably be somewhat lower than the true viscosity but may be taken as a useful approximation.

Temperature control

The hardness of butter varies very greatly with temperature, so that accurate control of temperature is the first essential in making measurements on the hardness of butter. In the present investigation an accurately controlled thermostat, which has been described elsewhere (13), was used. With this apparatus the temperature of butter samples could be kept during both storage and measurement within $\pm 0\cdot1°$ C. of the desired temperature. It is generally recognized that butter is slow in coming to equilibrium after a change in temperature. Some experiments were therefore made to determine the time of holding required to ensure that equilibrium was reached. A sample of butter was divided into two parts. One was held at 20° C. for 3 days and the other at 5° C. for a similar period. The samples were then cut into pieces of the size used for testing and immersed in water in the thermostat at 12·5° C. After 1 hr., when the samples had reached the temperature of the water, hardness determinations were made. The remaining portions of the samples were transferred to the storage cabinet in the thermostat and measurements were made on them at intervals. The hardness results expressed as load in grams required to produce a rate of cutting of 10^{-2} cm./sec. are given in Table 1.

The sample which had been held at a low temperature came to equilibrium fairly rapidly, but the one which had been held at a high temperature required at least 24 hr. to come to equilibrium. The technique decided on for the remainder of the investigation was to approach the temperature for measure-

ment from below and to hold the samples at the desired temperature for at least 24 hr. before commencing any measurements.

Table 1. *Hardness of butter samples, previously held at* 20 *and at* 5° C. *respectively, after holding for various times at* 12·5° C.

Time after bringing to 12·5° C.	Previous temperature	
	20° C.	5° C.
15 min.	385	455
3 hr.	415	434
20 hr.	442	448
7 days	—	450

Summary

Trials with the Scott Blair apparatus for measuring hardness of butter by compression of a cylindrical sample showed a lack of reproducibility due to splitting or crumbling of the sample. Investigation of alternative methods led to the development of an apparatus in which the resistance of butter to cutting by a wire is measured. The method gives results which are in general agreement with those obtained with the Scott Blair apparatus, but are much more reproducible. The effect of wire diameter and thickness of block of butter have been determined, and conditions for temperature control of the sample before and during the determination have been investigated.

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290. THE RHEOLOGY OF BUTTER

II. THE RELATION BETWEEN RATE OF SHEAR AND SHEARING STRESS. THE EFFECT OF TEMPERATURE AND OF REWORKING ON HARDNESS AND ON STRUCTURAL VISCOSITY

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(With 3 Figures)

During the last decade much progress has been made in linking up the elastic and plastic properties of various substances with their structure (1). The knowledge which has become available has made it possible for rheological studies to give a valuable insight into the structure of the material. Conversely, it has become possible to deduce in what way a modification of the structure would affect the physical properties.

Even where an investigation of a fundamental nature is not contemplated, a preliminary study of rheological properties is desirable to enable the best conditions for testing to be decided on. With materials such as butter, where the elastic deformation is of little importance compared to the plastic deformation, the plastic behaviour may be studied best by the determination of the flow-shear diagram. In many cases this diagram, which represents the relation between rate of flow and shearing stress, has a characteristic form which is related to the structure of the material.

Relation of rate of shear to shearing stress

For a liquid showing true viscous flow any increase in shearing stress (F) produces an exactly proportional increase in the rate of flow, or more precisely, rate of shear (V), so that if V is plotted against F a straight line is obtained as in curve 1 of Fig. 1. Where measurements are expressed in absolute units, V will represent the velocity in cm./sec. given by a force of F dynes/cm.^{2} to a surface at a distance of 1 cm. from another surface which is at rest. The slope of the curve dV/dF will be equal to $1/\eta$, where η is the viscosity. Hence $V = 1/\eta F$.

Where the size or number of suspended particles in the fluid becomes sufficient to form a structure across the channel of flow, viscous flow ceases and plastic flow begins. With a material of a truly plastic nature no flow takes place until the shearing stress reaches a value f (curve 2, Fig. 1) known as the 'yield value' for the substance. Any increase in stress beyond the yield value produces a proportional increase in the rate of shear and in this case again the slope of the curve is equal to $1/\eta$. The equation becomes $V = 1/\eta\,(F-f)$.

Where the suspended particles are built up into a more complex structure which does not collapse immediately on the application of a stress exceeding the yield value, a different type of flow takes place (Fig. 1, curves 3, 4). For such substances the rate of shear is not directly proportional to the shearing stress, and in many cases is found to vary as a power of the stress. The equations to curves 3 and 4 would then be

$$V = kF^n \quad \text{(curve 3)}, \qquad V = k\,(F-f)^n \quad \text{(curve 4)},$$

where k is a constant for the sample being measured. As the slope of the curve is not constant, the viscosity varies with the rate of shear. The index n, by giving a measure of the deviation from the ideal behaviour for which $n=1$, indicates the structural viscosity of the substance.

A further type of flow curve which is frequently encountered is that shown in Fig. 1, curve 5. Here no flow occurs until the stress reaches the value f_1 (the 'lower yield value'). With further increase in stress, quasi-plastic flow

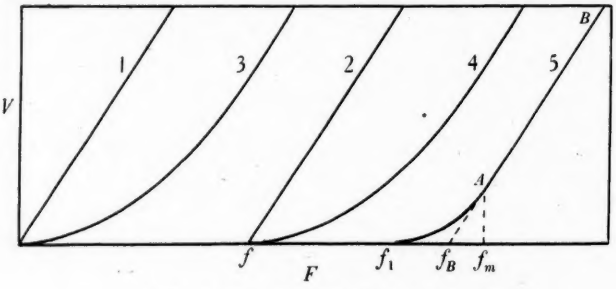

Fig. 1. Types of flow-shear curves.

takes place until the stress reaches the value f_m (the 'upper yield value') above which the flow is truly plastic. The portion AB of the curve is linear. Since neither the lower nor the upper yield value is sharply defined many workers have preferred to use the 'Bingham yield value' f_B obtained by producing the linear section of the curve till it cuts the F axis.

Kruisheer & den Herder(2) claim to have shown that butter gives a curve of type 5. Their measurements of hardness were made with a penetrometer, and the depth of penetration produced in a given time was taken as a measure of the rate of shear, while the load applied was taken as the shearing stress. When their results were plotted in this way curves of the shape of curve 5, Fig. 1, were formed. The Bingham yield value obtained by extrapolation was used as a measure of the hardness of the butter, and was found to agree well with graders' estimates of hardness. The deduction that butter exhibited a yield value was disputed by Pasveer(3), who criticized the technique used by Kruisheer & den Herder, pointing out that their temperature control was inadequate and that their apparatus was not suited to the measurement of very small penetrations. It appears to the present author that the rate of

shear of the butter would have been indicated more accurately by the time required for a given depth of penetration than by the depth of penetration in a given time. If the results had been expressed in this manner a different type of curve might have been obtained.

Scott Blair(4) has stated that 'Preliminary experiments with butter seem to indicate that...over a fair range of stress, the rate of shear varies as a power of the stress. This conclusion...gives a convenient way of expressing the data, since the log (rate of shear)/log (stress) curves are linear and their slope gives a measure of the extent of deviation of the material from truly fluid behaviour.' No figures were quoted, however, to support this conclusion.

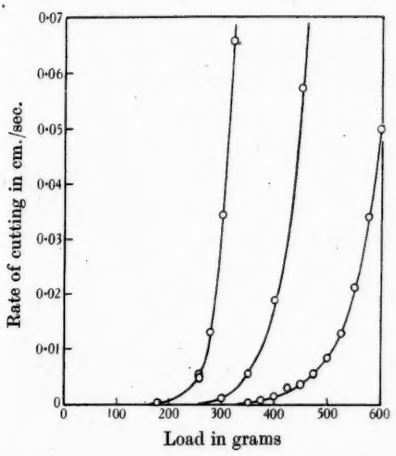

Fig. 2A.

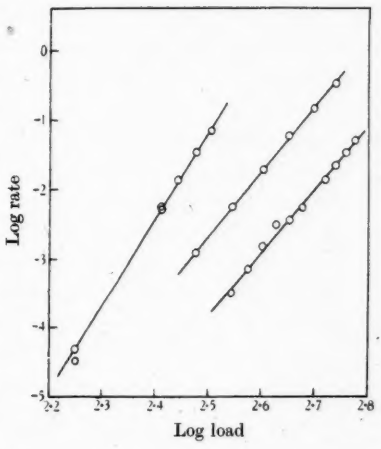

Fig. 2B.

Fig. 2A, B. Relation between load applied to wire and rate of cutting of butter.

EXPERIMENTAL RESULTS

The variation in rate of shear of butter with change in shearing stress was investigated by the sectility method(5) for a number of samples of butter over the widest possible range of stresses. The measurements were made at 12·5° C. The rate of cutting of the butter by the wire, expressed in cm./sec., was taken as a measure of the rate of shear of the butter, while the total load in grams applied to the wire was taken as the shearing stress. Curves obtained for three typical samples are shown in Fig. 2A. These curves, although covering a wide range of rates of shear and including the whole of the range studied by Kruisheer & den Herder, do not become linear at the higher stresses. In Fig. 2B the curves have been redrawn as log (rate of cutting)/log (load) curves. The curves of Fig. 2B are linear over their whole range. This relation has been found to hold for all the samples examined (over 200). The con-

clusion of Scott Blair is therefore supported. The curves for rate/load are thus of the type of curve 3 in Fig. 1 and may be represented by the equation

$$V = kF^n.$$

The value of n may be determined from the slope of log/log graph.

The fact that the rate/load curve is of the form $V = kF^n$ shows that at 12·5° C. butter does not exhibit a yield value.

The implication of this conclusion is that butter is a non-Newtonian liquid and not a solid, and that under the smallest loads some flow would be observed if a long enough time were allowed. The shape of the curve is such, however, that a 'practical yield value' is exhibited. This term is defined by Houwink(1) as follows: 'The tension at which flow becomes observable practically, under the usual conditions of test.' The practical yield value will of course vary with the rate at which the test is carried out. The practical yield value provides a convenient method of expressing the hardness of butter. For the purposes of the present experiments it is taken as the load in grams required to produce a rate of cutting of 10^{-3} cm./sec., the lowest rate which can conveniently be observed with the sectility apparatus. The practical yield value should provide a measure of 'stability' or 'standing up quality' of butter, i.e. the ability of a pat of butter to maintain its shape and resist flow under the influence of its own weight. Other tests carried out at a slow rate, e.g. the sagging beam test used by Coulter & Combs(6), would be expected to give similar indications.

Where it is desired to measure the 'spreading capacity' of butter a different index must be used. In spreading butter on bread the knife is moved with a speed of from 10 to 100 cm./sec., and the rate of shear of the butter will be correspondingly high. If the value of the slope n were the same for all butter samples it would be permissible to use measurements made at a slow rate as an index of spreading capacity. It was found, however, that the value of n for different samples at 12·5° C. may vary between 8 and 15. Two samples with the same practical yield value might therefore differ considerably in their resistance when spread or tested at a high rate. Measurements to determine spreading capacity should be made at a flow rate similar to that actually used in spreading butter on bread. It has not been possible to use such a high rate with the sectility apparatus, but, in order to obtain an index of spreading capacity, the load required to produce a rate of cutting of 10 cm./sec. has been determined by extrapolation assuming that there is no appreciable deviation from the linear form of the log/log curve up to this rate of flow.

The form of the curve for (rate of strain)/(shearing stress) for butter shows that butter possesses a very high structural viscosity. It has been observed with other substances that a high structural viscosity as indicated by a value of n much greater than 1 is usually accounted for by the presence of non-spherical dispersed particles. Solutions in which the dispersed particles or molecules are more or less spherical show values of n approximating to 1. This is in accord with the idea that spherical particles cannot be built up into

such a rigid structure as can long rod-shaped particles. The high values of structural viscosity observed in butter could hardly be accounted for on the basis of the theory put forward by King(7) that butter is a dispersion of the fat globules and water droplets in liquid fat. It is difficult to imagine a series of fat globules linked together sufficiently firmly to give a structure of any rigidity. High values of structural viscosity could possibly be attained in the presence of long chain or complex molecules (e.g. glycerides of the higher fatty acids) but a more likely explanation is the presence of needle shaped crystals of fat in the continuous phase. These crystals, by forming an interlaced network enclosing the existing fat globules and water droplets, would create a structure of considerable rigidity.

Effect of temperature on hardness

Determinations of hardness of butter at different temperatures were made as follows:

Seven samples of butter which had been made at different times in one season from September to April and which covered the whole range of hardness likely to be encountered in New Zealand butter were selected. The samples made early in the season had been held in cold storage at −9·5° C. (15° F.) for some months. All samples were maintained at 2° C. (35° F.) for at least a month before the measurements were made so that they might all come to equilibrium under the same conditions. The samples were then transferred to the thermostatic cabinet and held at 6° C. for 3 days. Measurements were made at this temperature on portions of all the samples. The temperature of the thermostat was then raised to 8° C. and the samples allowed to remain overnight to come to equilibrium at the higher temperature. After a further series of measurements the temperature was raised another 2° C. This process was repeated till the final series of measurements at 18° C. was made. The results of the determination are shown in Table 1, and the hardness values have been plotted against temperature in Fig. 3.

Table 1. *Variation in hardness of butter with temperature*

Sample no.	Churning date	Hardness 6°	8°	10°	12°	14°	16°	18°
1	7. ix	—	273	—	160	135	—	60
2	21. ix	—	457	—	320	255	—	86
3	19. xii	711	573	474	395	283	185	102
4	30. i	656	575	460	391	308	182	146
5	8. ii	682	617	515	447	339	269	174
6	21. iii	611	533	454	353	273	198	110
7	2. iv	461	413	375	294	243	155	88
		Slope n						
		6°	8°	10°	12°	14°	16°	18°
1		—	14·2	—	12·8	10·5	—	—
2		—	11·0	—	10·4	8·7	—	—
3		12·1	11·6	10·2	8·9	8·9	6·6	5·3
4		11·6	15·0	10·2	8·8	8·4	6·3	5·7
5		13·2	12·0	9·8	9·3	7·4	7·6	7·5
6		15·8	12·5	11·1	8·9	7·5	8·0	8·0
7		12·7	10·8	12·1	8·1	8·3	8·1	7·1

The hardness (practical yield value) of the butters showed a steady fall with increase in temperature. The rate of decrease of hardness was not the same for all the samples, so that their order of hardness changed as the temperature was increased.

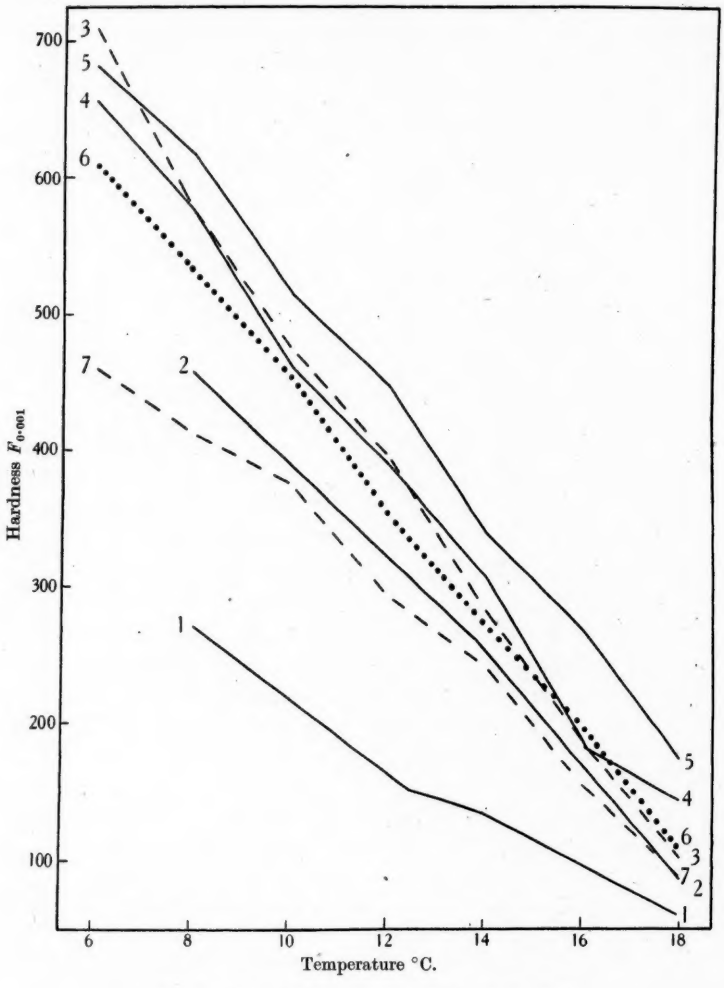

Fig. 3 Effect of temperature on hardness of butter.

It was found that the value of the structural viscosity n tended to decrease as the temperature was raised. This reduction of structural viscosity could be explained by the melting or solution of crystals of fat from which the structure was built up.

Effect of reworking butter

Since the hardness of butter depends so much on structural viscosity, it is only to be expected that working of the sample should have a pronounced effect on the hardness.

A sample of butter which had been kept at 12·5° C. was thoroughly worked at that temperature. Determinations of hardness were made immediately after reworking and at intervals later. The results are given in Table 2.

Table 2. *Effect of reworking*

	Hardness	n
Before reworking	398	8·9
Immediately after	93	8·9
4½ hr. after	103	8·9
22 hr. after	120	6·8
3 days after	146	7·7
6 days after	162	7·9
37 days after	278	—

It was found that reworking decreased the hardness to about a quarter of the original value. There was a slow recovery on standing, but even after several weeks the hardness was still much below the original value. There was surprisingly little change in the value of n with reworking. Apparently although the working breaks down the structure to such an extent as to produce a very marked reduction in hardness, there is still a sufficiently complex structure remaining to show structural viscosity and to permit a further breakdown of structure with flow.

SUMMARY

It has been shown that the rate of shear of butter when cut by a wire increases as a power of the load applied to the wire. The values of this power are high (index $n=8$ to 15, average 11 at 12·5° C.) and show a high structural viscosity in the butter. There is no indication that butter exhibits a true yield value, but the (rate of shear)/(load) curve is such that a practical yield value is found. The practical yield value (defined as the minimum load required to produce a measurable rate of flow) provides a convenient measure of the hardness of butter. The viscosity of butter decreases rapidly as rate of shear is increased, but the rate of decrease varies from one sample to another. Measurements of spreading capacity should be made at rates of shear comparable with those which occur when butter is spread with a knife.

The effect of temperature over the range 6–18° C. on the hardness and structural viscosity of a number of butter samples has been investigated. The effect of reworking has also been studied.

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291. THE RHEOLOGY OF BUTTER

III. THE EFFECT OF VARIATION IN BUTTERMAKING CONDITIONS ON THE HARDNESS OF THE BUTTER

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The body and texture of butter are influenced to a considerable extent by the methods of manufacture. It is not surprising, therefore, that a large number of investigations have been made with the object of finding the optimum conditions for buttermaking, or of discovering methods of eliminating faults such as crumbliness or lack of spreading capacity. In a number of instances measurements of hardness have been used to provide an index of the body or the spreading capacity of the butter. A considerable degree of success has been achieved in these researches, but there is such a wide variation in conditions between one country and another, particularly in the composition of the butterfat, that results obtained in one locality are frequently inapplicable in another.

It is not proposed to review the extensive literature on the subject (a summary has been given in a paper by Coulter & Combs(1)), but the most generally accepted findings will be briefly stated as follows: Rapid cooling of cream after pasteurization and low temperature holding or churning of cream all tend to produce harder butter. The use of very cold wash water tends to make the butter softer.

These conclusions do not represent a unanimous verdict and it cannot be assumed that they will apply under New Zealand conditions. The only investigation on the subject made in the Dominion is that of Valentine & Sargent(2) who confined their attention to a study of the effect of rate of cooling of cream after pasteurization. Using an extrusion pressure apparatus for measuring the hardness of the butter, they showed that 'shock' cooling of the cream on a direct expansion cooler gave a harder butter than did slow cooling in a refrigerated vat. The differences, however, were no greater than the day-to-day variations in the hardness of the butter.

The present investigation has covered all the main stages in the buttermaking process from the pasteurization of the cream to the working of the butter. Except where the particular experiment made modifications necessary, the manufacturing technique was as follows:

Cream purchased from a commercial factory was bulked in a mixing vat on receipt at the experimental factory. The cream was pasteurized in a Murray Vacreator in which it was heated to 200° F. by direct contact with steam and was then subjected to steam distillation in two stages, first at 16–17 in. and

then at 26–28 in. of vacuum. On leaving the Vacreator the cream passed over water and direct expansion coolers and was collected in cans which were emptied in turn into each of two or three refrigerated holding vats. In this way the effect of any variation in pasteurizing or cooling temperature was minimized. Starter cultures were not used in ripening the cream. After holding at 35–45° F. for approximately 18 hr. the cream was churned in a small internal worker churn with a capacity of 120–180 lb. of butter. Parallel churnings were made under as nearly the same conditions as possible. Samples of the butter taken after churning were held at 2° C. (35° F.) for 5 or 6 days and then at 12·5° C. for 2 days before measurements of hardness were made. The bulk of the butter was stored at −9·5° C. (15° F.) for 3–6 months after which the hardness was again determined. In a few instances measurements of hardness were made at 18° C. as well as at 12·5° C. In the earlier experiments the Scott Blair apparatus(3) was used in determining the hardness, while in the later experiments the measurements were made with the sectility apparatus described in the first paper of this series(4). Results with the Scott Blair apparatus are reported as load in pounds required to compress a cylinder of butter 4 cm. in diam., from a height of 2 cm. to a height of 1·21 cm. in 60 sec. Results with the sectility apparatus are given as loads in grams required to produce rates of cutting of 10^{-3} cm./sec. ($F_{0·001}$) and of 10 cm./sec. (F_{10}). The former figure ($F_{0·001}$) indicates the 'practical yield value' and the latter (F_{10}) the 'resistance to spreading'.

Values of the slope n (see previous paper(5)) are also recorded for each sample.

The results are summarized in Tables 1–6.

Table 1. *Effect of type of pasteurizer on hardness of butter*

A. Scott Blair method. Measured at 12·5° C.

Churning date	Before storage			After storage		
	Flash	Vacreator	A.P.V.	Flash	Vacreator	A.P.V.
28. iii	22·1	20·3	20·8	25·2	24·0	25·6
30. iii	19·4	18·2	17·5	23·5	24·2	23·9
5. ix	12·8	12·9	—	—	—	—
7. ix	9·6	9·6	—	—	—	—
Average	16·0	15·3	—	24·4	24·1	24·8

B. Sectility method

Churning date	Method	Before storage 12·5° C.			After storage 12·5° C.			After storage 18° C.		
		$F_{0·001}$	F_{10}	n	$F_{0·001}$	F_{10}	n	$F_{0·001}$	F_{10}	n
5. ix	Flash	202	473	10·8	204	472	11·0	69	182	9·5
	Vacreator	202	485	10·5	180	422	10·8	73	195	9·4
7. ix	Flash	147	340	11·0	146	303	12·6	53	133	10·0
	Vacreator	145	345	10·6	155	348	11·4	50	126	10·0
Average	Flash	175	407	—	175	388	—	61	158	—
	Vacreator	174	415	—	168	385	—	62	161	—

Table 2. *Effect of rate of cooling of cream on hardness of butter*

A. Scott Blair method. Measured at 12·5° C.

Churning date	Before storage			After storage		
	Vat	A.P.V.	D.E.	Vat	A.P.V.	D.E.
18. x	15·8	18·0	17·5	16·0	18·4	18·6
20. x	17·3	19·5	18·5	18·0	19·4	19·3
21. iii	18·5	25·5	24·9	21·2	26·2	26·4
23. iii	18·5	23·3	23·8	20·4	25·2	26·0
Average	17·5	21·6	21·2	18·9	22·3	22·6

B. Sectility method

Churning date	Method	Before storage 12·5° C.			After storage 12·5° C.			After storage 18° C.		
		$F_{0·001}$	F_{10}	n	$F_{0·001}$	F_{10}	n	$F_{0·001}$	F_{10}	n
30. viii	Vat	134	332	12·0	166	447	9·3	66	178	9·3
	D.E.	180	404	11·4	180	511	8·8	80	200	9·8
1. ix	Vat	146	301	12·7	145	335	11·0	51	126	10·2
	D.E.	163	340	12·5	177	395	11·5	67	179	9·4
Average	Vat	150	317	—	156	391	—	59	152	—
	D.E.	172	372	—	179	453	—	74	190	—

Table 3. *Effect of temperature of holding cream on hardness of butter*

Sectility method

Churning date	Temp. ° C.	Before storage 12·5° C.			After storage 12·5° C.			After storage 18° C.		
		$F_{0·001}$	F_{10}	n	$F_{0·001}$	F_{10}	n	$F_{0·001}$	F_{10}	n
19. ix	35	225	532	10·7	270	697	9·7	76	209	9·1
	40	234	532	11·2	251	649	9·7	66	168	9·9
	45	214	498	10·9	256	610	10·6	71	242	7·5
21. ix	35	251	568	11·3	297	865	10·2	73	203	9·0
	40	263	590	11·4	306	842	11·2	62	196	8·0
	45	258	592	11·1	306	874	10·3	84	211	10·0
Average	35	238	550	—	284	781	—	75	206	—
	40	249	561	—	279	746	—	64	182	—
	45	272	545	—	281	722	—	78	127	—

Table 4. *Effect of churning temperature on hardness of butter*

Scott Blair method. Measured at 12·5° C.

Churning date	Before storage				After storage			
	36° F.	40° F.	44° F.	48° F.	36° F.	40° F.	44° F.	48° F.
4. x	14·7	14·6	14·4	—	—	—	—	—
6. x	16·3	15·4	15·5	—	—	—	—	—
11. x	19·0	19·0	18·5	—	—	—	—	—
13. x	19·7	20·8	19·5	—	—	—	—	—
8. xi	—	19·2	20·5	—	—	19·5	22·2	—
10. xi	—	24·5	25·8	—	—	25·0	27·7	—
15. xi	—	—	20·5	19·8	—	—	22·6	22·2
17. xi	—	—	25·7	25·0	—	—	27·5	25·7
7. iii	—	24·7	23·5	26·0	—	26·6	27·3	30·3
9. iii	—	26·9	27·3	28·8	—	30·3	30·5	30·3

Table 5. *Effect of wash-water temperature on hardness of butter*

A. Scott Blair method. Measured at 12·5° C.

Churning date	Before storage			After storage		
	35° F.	45° F.	55° F.	35° F.	45° F.	55° F.
20. ix	12·5	12·6	13·0	13·0	13·7	15·3
22. ix	12·4	12·5	13·0	14·0	14·6	14·7
14. iii	24·2	24·1	24·0	26·5	26·9	27·8
16. iii	23·4	25·5	23·3	27·7	28·1	27·9
12. ix	14·7	14·3	14·5	—	—	—
14. ix	13·0	12·9	13·4	—	—	—
Average	16·7	17·0	16·9	20·3	20·8	21·4

B. Sectility method

Churning date	Temp. ° C.	Before storage 12·5° C.			After storage 12·5° C.			After storage 18° C.		
		$F_{0·001}$	F_{10}	n	$F_{0·001}$	F_{10}	n	$F_{0·001}$	F_{10}	n
12. ix	35	227	528	10·9	236	496	12·6	81	212	9·6
	45	237	540	11·2	256	504	13·6	87	246	8·8
	55	237	536	11·3	246	537	11·8	84	224	9·4
14. ix	35	200	431	12·0	246	500	13·0	76	231	8·3
	45	211	454	12·0	240	546	11·2	78	237	8·3
	55	216	454	12·4	251	594	10·7	84	255	8·3
Average	35	214	480	—	241	498	—	79	222	—
	45	224	497	—	248	526	—	83	242	—
	55	227	495	—	249	566	—	84	240	—

Table 6. *Effect of amount of working on hardness of butter*

Scott Blair method. Measured at 12·5° C.

Churning no.	Before storage			After storage		
	Under worked	Normal	Over worked	Under worked	Normal	Over worked
1–8–11	19·2	19·2	18·4	20·8	19·5	20·8
2–8–11	20·5	20·5	21·5	21·2	22·2	23·0
1–10–11	26·0	24·5	22·5	26·3	25·0	25·6
2–10–11	26·5	25·8	24·7	28·3	27·7	27·8
1–15–11	19·9	20·5	20·3	22·5	22·6	24·0
2–15–11	19·6	19·8	19·5	22·4	22·2	22·3
1–17–11	22·5	25·0	22·6	24·7	25·7	28·1
2–17–11	25·1	25·7	25·5	27·4	27·5	27·8
Average	22·4	22·6	21·9	24·2	24·1	24·9
1–4–4	—	18·1	18·5	—	16·2	16·2
2–4–4	—	20·5	23·0	—	21·5	21·5
3–4–4	—	23·3	22·8	—	22·5	22·5
1–6–4	—	18·7	18·2	—	15·2	15·2
2–6–4	—	21·1	23·0	—	20·9	20·9
3–6–4	—	22·1	22·5	—	21·6	21·6
Average	—	20·6	21·3	—	19·7	19·7

Type of pasteurizer (Table 1). A comparison was made between a Tandem Flash Pasteurizer, a Vacreator and an A.P.V. plate pasteurizer. Pasteurizing temperatures in the first two machines were normally 200° F., while in the A.P.V. pasteurizer the cream was held at 160° F. for 29 sec.

The butters produced showed no consistent differences in hardness.

Rate of cooling after pasteurization (Table 2). In this experiment one portion of the cream was cooled to a temperature of about 70° F. on a water cooler, and was then run into a holding vat in which the temperature was slowly reduced to 36–40° F. The minimum temperature was reached in about 5 hr. The remainder of the cream was cooled to the same temperature by passing over a direct expansion cooler or through an A.P.V. brine cooler.

The butter made from the slowly cooled cream was invariably softer than that made from the rapidly cooled cream. There was no significant difference in hardness of butters made from cream cooled with the D.E. cooler and with the A.P.V. cooler respectively.

Temperature of holding after pasteurization (Table 3). The pasteurized cream was divided into three lots which were held respectively at 35, 40 and 45° F. Before churning the temperature was always brought to 40° F.

Differences in hardness of the butters were small and showed no consistent variation.

Churning temperature (Table 4). The cream was divided after pasteurization and cooling and each portion was held overnight at the temperature at which it was to be churned.

There was very little variation in hardness with variation in churning temperature.

Wash-water temperature (Table 5). Churnings from the same batch of pasteurized cream churned under the same conditions were washed with water at different temperatures.

Here again the differences in hardness of the butter were very slight, but were more consistently in the same direction. There appeared to be some tendency for the butter washed with the colder water to be softer than that washed with water at a higher temperature. The differences, however, were too small to be of any practical significance as far as spreading capacity is concerned.

Amount of working (Table 6). A quantity of cream sufficient to make three 56 lb. boxes of butter was churned under normal conditions. After the butter had received a light working, enough was removed to pack one box. The working was then resumed till the butter had had a normal amount of working and a further lot was removed. The third portion was worked till the butter adhered to the churn and working could not be continued.

The hardness of the butters, particularly after storage, was very little affected by the wide differences in the amount of working.

Discussion

Of the various factors investigated, the only one which had any marked influence on the hardness of the butter was the rate of cooling after pasteurization. This effect of rate of cooling has already been observed in New Zealand by Valentine & Sargent (2), as well as by workers in other countries. It is the usual practice in New Zealand to pass the cream over a direct expansion

cooler after pasteurization so that there will be a tendency for the butter to be somewhat harder than when the final stages of cooling are carried out in a refrigerated vat.

Coulter & Combs(1), working at Minnesota, found the temperature of the wash water to be an important factor in controlling the body of the butter and particularly in preventing crumbliness in hard winter butter. The use of low temperature wash water was found to give a softer butter with improved spreading characteristics. The results of the present investigation indicate that under New Zealand conditions, although there is a slight tendency for the butter produced with cold wash water to be softer, the difference is extremely small.

Coulter & Combs have also noted that the use of low holding or churning temperatures causes some increase in the hardness of the butter. In the present investigation the exposure of the cream to low temperatures at these stages has not been found to produce any consistent increase in the hardness of the butter.

SUMMARY

An investigation of the effect of type of pasteurizer, rate of cooling after pasteurization, temperature during holding and during churning, temperature of wash water and amount of working showed that of these factors the only one which had any marked and consistent effect on the hardness of the butter was the rate of cooling of cream after pasteurization, rapidly cooled cream yielding harder butter. The use of low temperature wash water tended to reduce the hardness of the butter but the effect was small. The other factors had no consistent effect on the hardness of the butter.

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

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SECTION B: BACTERIOLOGY AND MYCOLOGY APPLIED TO DAIRYING

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I. MILK CONTROL

General technique and sampling

A second edition of Chalmer's useful *Bacteria in Relation to the Milk Supply* (1), and a seventh edition of the American *Standard Methods for the Examination of Dairy Products* (2), have appeared, the latter describing the new American standard medium.

The importance of common details in bacteriological work has been rather neglected in the past, and Barkworth (3) has continued tests on hot-air sterilization, showing that 2½ hr. heating at 160° C. is necessary to destroy spores of *B. subtilis*; he recommends 3 hr. heating for routine purposes.

The question of control of samples before testing is not less important than the laboratory work, and Mattick, Barkworth & Hoy (4) have found that expanded rubber is an excellent insulating material for boxes containing milk samples. They showed by thermo-couple measurement that a multi-cell box was desirable and recommend a box containing ten 6 oz. bottles in two rows of five. Earlier, Barkworth (5) had found that expanded rubber was the best of the insulating materials tested, from 8 to 10 hr. being required for the samples to reach atmospheric temperature.

The same author (6, 7) has also compared the Mattick & Hoy 20 ml. rinse for milk-bottle counts with one of 90 ml. (in America 100 ml. is used), and concludes that the former is more likely to distinguish 'really clean' and 'nearly clean' bottles, giving, as it does, a higher colony count per ml. rinse water. His results also confirm the conclusion of Mattick & Hoy that a coliform test is not worth while when a high standard for total count is required.

An important objection to the use of the plate count for routine control work is the expense involved. Hucker & Haynes (8) have recommended the Burri slant technique for determining the *relative* numbers of bacteria in milk and for the control of plant processes. It is also of great value for udder samples, especially if the milk is first incubated. Thus of 1372 samples only eighteen revealed streptococci by direct

microscopic examination, but over 80% were shown to contain streptococci after incubation. Burri(9) has also re-emphasized the advantages of surface colonies for identification and the study of dissociation, antagonism and autolysis.

Colony count methods

Lind(10), reporting on a symposium on the new tryptone-glucose-extract-milk agar, states that all laboratories experienced an increase in size of colony with the new medium, and some claimed that pin-point colonies were eliminated. Improvement in detection of thermophilic and thermoduric organisms was noted, but findings on the type of milk which gave the most marked difference with the new medium were variable, most laboratories claiming that the greatest increase occurred with high-count milk. Considerable trouble was experienced with precipitates in the medium, and most workers preferred fresh skim milk to the dehydrated form. One laboratory reported that sterilizing at 15 lb. pressure for 20 min. and holding the melted agar at 50° C. eliminated the precipitation problem. Dixon(11), in a discussion of the symposium, emphasized the value of the new medium in showing up the 'borderline cases' by growing bacteria associated with unclean utensils and inadequate cooling. Abele & Damon(12) have similarly compared the old and new media by testing 1000 samples of raw milk. The average percentage increase in arithmetic means for the nine arbitrary grades varied from 9·3 to 42·6%, with a total range of −55 to +1718% when single samples were considered. They conclude that the relationship of the counts is so irregular that it is impossible to express one count in terms of the other, but show that the use of the new medium would only have resulted in altering the grade of raw milk in 7·4% of cases (2·6% in the case of pasteurized milk).

Nelson(13, 14), working with rather small numbers of samples, found that the ratio of counts (log averages) on the new to that on the old medium at 37° C. was 1·36 for raw milk, 1·82 for pasteurized milk and cream, and 1·35 for ice cream. At 32° C., in the case of pasteurized milk only, the ratio was appreciably less than that at 37° C. Counts obtained with the Bower & Hucker medium did not differ significantly from those on the new medium. Both the new and the Bower & Hucker medium gave larger colonies than did the old medium. Abele(15) has analysed the results of a co-operative experiment in which 335 samples of various types of milk were plated in the old and new media, and also in the Medical Milk Commission's (M.M.C.) medium, and incubated at 37 and 32° C. The new medium gave an average increase in count over the old of 48%, the M.M.C. medium giving an increase of 36%. All media gave higher counts at 32 than at 37° C., average increases of 60–70% being found. The use of the new medium at 32° C. gave a cumulative increase of 114%. Marcus(16) concludes from his data that the use of the new medium at 32° C. need not entail any alteration in the standard set for the old medium at 37° C. With pasteurized milk significant increases were sometimes obtained, and the author confirms that when high counts are the result of poor cooling, post-pasteurization counts are usually low, but the reverse holds when dirty utensils are the cause of the high counts. Broadly speaking, the new medium gave counts about equal to the microscopic count in the case of raw milk.

Foltz & Bushnell(17) report that lactic acid bacteria, especially the pyogenic streptococci, give improved growth in the new medium. It is therefore a better medium for use in mastitis testing. Farrell(18) has also reported that Hucker's, and Safford & Stork's medium, and one containing Lemco, gave higher counts than the old standard medium. Foltz & Nelson(19) have also reported that the new medium gives better growth with pathogenic and saprophytic streptococci and also with *L. casei* and *L. acidophilus*, but that *Str. thermophilus* and *L. bulgaricus* did not grow on either medium.

Redowitz(20) has described a simple tube method for making counts of non-motile bacteria. He uses semi-solid media (0·15% agar) and prepares his first dilution by adding 1 ml. of the culture to 99 ml. of diluent. He claims that 18 hr. incubation is sufficient to produce easily visible colonies; the temperature is not given. Davis & Newland(21) have investigated different concentrations of agar for semi-solid media for milk and churn tests and conclude that 0·3% is most suitable. Counts comparable with the standard plate count at 37° C. were obtained, some organisms forming large colonies in 18 hr. and the remainder requiring 48 hr. to form easily countable colonies.

Myers & Pence(22) regard the laboratory pasteurization count as a sounder criterion of the true sanitary quality of a milk than either the raw-milk count or the methylene-blue test. In view of the well-known disadvantages of smear techniques, these writers advocate mixing standard loopfuls of pasteurized milk with the new medium and 'sloping' in oval tubes. An important step was the shaking of the pasteurized and cooled milk; without this treatment lower counts were obtained. Tryptone-glucose-extract agar without skim milk was used, as the milk was troublesome and had no significant effect on the count. The authors claim that their technique is as fast as the direct microscopic count, twice as fast as the ordinary plate count, and gives much more information.

A simple differential medium has been described by Davis(23) containing, in addition to peptone, Lemco and yeastrel, 1% lactose, 1% separated milk and 1% chalk, with 0·002% brom-cresol purple as indicator. Acid-formers are readily detected by zones of clearing in the chalk and, of these, lactic-acid bacteria are readily differentiated from coliforms and staphylococci by flooding with 1% hydrogen peroxide. It is particularly useful for testing freshly taken single quarter samples for mastitis, especially if blood is not available. Barkworth & Davis(24, 25) have tried a large number of different combinations of various concentrations of peptone, Lemco and yeastrel in an endeavour to find a satisfactory medium containing only very little peptone. They conclude that a medium with 0·2% peptone, 0·1% Lemco and 1% separated milk gives counts not significantly different from those given by yeastrel agar(26) and report that the colonies are practically as large.

Robinson(27) has confirmed that 'spreaders' may be controlled by using smaller (10 ml. or less) amounts of medium.

Direct microscopic count

Although not much used in this country the direct count has always received attention in America and there is evidence that this is increasing.

van der Burg, de Kadt & van Krevèld(28) have investigated some of the technical and statistical problems involved in the direct count. They preferred pipettes to loops, could not detect any appreciable effect due to shaking, and obtained a Poisson distribution in the numbers of bacteria in fields. The mean error in counting was related to the number counted. Hanks & James(29) found that the distribution of organisms in the usual smear was very irregular, counts varying from five at the periphery to 333 in the centre. Increase of fixative from 0·01 to 0·02 ml. lowered the counts. It is suggested that, where from six to twenty bacteria occur per field, twenty-two fields should be counted; when over twenty bacteria are present per field, eleven should be counted. Antunes Tropa & Marques Esteves(30) compared the Breed-Brew and Skar methods on 107 samples of raw milk. They do not agree with the findings of other workers that the Skar is superior to the Breed method. The latter was, on the whole, preferred because of speedier working and ability to detect cells. Little difference in count was found.

Fox, Turney & Bryan(31) have emphasized the fact that the direct count not only compares very favourably with the plate count, but also affords a means for determining the *cause* of poor quality in milk. They classify causes as (1) dirty utensils,

(2) poor cooling, (3) dirty cows, etc., (4) dust contamination, (5) poor production, (6) mastitis and (7) excess cells. They give relationships between various tests and the causes of trouble. For milks not reducing methylene blue in 5½ hr. (6) and (7) are the most important. June to August showed the highest incidence of failures to the methylene-blue test. An attempt to define causes of failure on milks having counts of >100,000 showed that (1) accounted for 22%, (2) 18%, (3) 16%, and whereas (6) accounted for but 6%, (7) was responsible for 22%. Sediment appeared to have no relationship to count. Macy(32) has published some valuable figures for the microscopic examination of pasteurized milk. Of samples as delivered to the consumer, the microscopic count was, on the average, eighty-two times the plate count (old medium). Commercial plant samples gave an average ratio of 98 and laboratory-pasteurized samples 113. There is a general tendency towards higher microscopic counts when the thermophile (55° C.) count is high. Indeed, the direct count may be used to show the *increase* in count as a result of growth of thermophiles during pasteurization when plating methods fail to do so. Allen(33) has described a method suitable for all liquid cultures, and Boynton & Hiscock(34) have given further evidence of the value of the direct count.

Methylene-blue test

Johns(35), in a comprehensive paper on various technical points influencing the methylene-blue test, has shown that refrigerated and dirty milks reduce rather faster at temperatures below 37° C., but that there is no case for altering the present temperature. Increasing the concentration of dye from 1 in 700,000 to 1 in 300,000 and non-inversion, both increased the reduction time, the former by about 20% and the latter to a very variable extent. Johns favours preliminary incubation at 13° C. for 18 hr., as this lowers the reduction time and gives a better measure of keeping quality. He gives the following mean relationships for reduction times in hours:

No preliminary incubation		Preliminary incubation at 13° C. for 18 hr.
Tubes not inverted	Tubes inverted every 2 hr.	Tubes inverted every 2 hr.
11	7½	4½
8½	6	2½
5	4	1
2	2	¼

He favours inversion at 2, 4 and 6 hr. only, and suggests these as convenient times for inspection and grading.

In a later paper(36) the same author states that, with 1 in 200,000 thiocyanate, the potential falls sooner but the dye reduces on the average 30 min. later than with the chloride (1 in 700,000). This is due to the fact that the former dye decolorizes at a lower potential.

Williams, Davies & Thomas(37) have compared the old and new modified techniques and found little difference with milks reducing within 5½ hr. but a marked quickening with better quality milks. Phillips, Ashton, Simons & Thomas(38) have studied the variability of the test and consider that the magnitude of the variation is controlled by the intervals between examination, the number of workers, the quality of the milk and the personal factor. With the ordinary technique (half-hourly inspection) no variation was found with milks reducing in 5 hr., but slight variation was found with better milks. When one worker examined good milks every 5 min. in quintuplicate the mean coefficient of variation was less than 1%, but when four workers examined good milks in winter the coefficient was 6%. Lower variations were found in summer.

The importance of storage conditions cannot be too greatly stressed, and Powell, Jenkins & Thomas(39) have found that milks held overnight at 40° F. gave an average

reduction time that was 3½ hr. longer than when held at 60° F., the actual differences ranging from 54 min. in the case of dirty milks to 4⅓ hr. for good milks. These workers consider that a holding temperature of 40° F. is too lenient in winter and one of 60° F. too drastic in summer. They suggest that samples should be sent in well-insulated containers and held at 50–55° F. in the laboratory. Garrison(40) has compared temperatures from 32 to 47° C. for the methylene-blue test and finds that the best temperature on the whole was 42° C. The statement that the reduction time was shortened by adding 1% lactose is surprising. Formate increased the reduction time and sucrose had no effect. Ciani(41) has studied the methylene-blue test with pasteurized milk. He found a linear relationship between reduction time at 37° C. and keeping quality but not between plate count and keeping quality. Wide variance was found in the plate count but not in the methylene-blue and keeping-quality tests. He failed to get a reasonable relationship between methylene-blue reduction time at 55° C. and thermophilic count, but considers that a methylene-blue time of > 12 hr. at 55° C. indicates reasonable freedom from thermophiles.

Davis & Lines(42) have studied flora-type distribution in summer and in winter and found that the proportion of acid-coagulating-proteolytic-gas-producing types increased markedly in June–September. Acid-non-coagulating types were predominant in good milk but were poor reducers of methylene blue. These authors consider the proteolytic types to be strong oxygen absorbers and to play an important role in reduction of methylene blue (but cf. Hobbs(43)). Acid-coagulating, alkaline and inert types did not affect reduction appreciably. Of 25,000 cultures in litmus milk 6·3% were acid, coagulating; 55·8% acid, non-coagulating; 11·6% acid, coagulating, proteolytic, gas-producing; 16·6% acid, proteolytic; 2·7% alkaline; and 2·8% inert; while 4·4% failed to grow.

Résazurin test

A feature of the last two years has been the growth of interest in this test. Little(44) compared the methylene-blue and resazurin tests on 1434 samples of milk and also carried out microscopic counts on 304. He found no advantage in refrigerating the samples at 40° F. for 4 hr. before testing. Taking as standards a direct count of 200,000 or less as *good*, and one of over 400,000 as *bad*, and a methylene-blue reduction time of 5 hr. or less as *bad*, resazurin end-points of purple-pink and pink were classified after 1, 2 and 3 hr., and the results of all these tests compared. He found that 5·6% reduced methylene blue but passed the count, 6·3% passed on methylene blue but failed on count; similar figures were obtained taking for resazurin end-points of purple-pink after 2 hr. or pink after 3 hr. The general observation that mastitis milks and those containing weakly reducing organisms reduce rapidly at first and then experience a lag was confirmed.

Johns(45) has shown that the test can be shortened by inverting the tubes at intervals of 2 hr. or less, and also by preliminary incubation at 55° F. for 18 hr. Davies, Thomas & Thomas(46), comparing the resazurin test with the modified methylene-blue test, found that resazurin required on the average 35 min. longer for complete reduction. They obtained an r value of +0·87 for complete reduction of resazurin and one of +0·66 for the purple-pink stage. They obtained the usual proportion of discrepancies, 11% of the samples reducing to purple-pink in 1 hr. but failing to reduce methylene blue in 7 hr. Later, Thomas & Davies(47) studied a large number of summer and winter samples. They found that 81% passed or failed both the resazurin (purple-pink in 1 hr.) and the methylene-blue tests (accredited standards) and that 10% passed the resazurin but failed the methylene-blue test, these having low bacterial but high cell counts. Relatively more failed methylene blue in summer and resazurin in autumn and winter. The authors attribute this latter finding to the high proportion of late-lactation and post-colostrum milk. The phenomenon was accentuated in January.

Russell, Palmer-Jones & Moir(48) have studied and confirmed the value of the resazurin test for mastitis testing. In their hands the test detected 73% of staphylococcal infections, 72% of streptococcal, 12% of 'micrococcal' and gave 8% false positives, of which only 2% were inexplicable, the remaining 6% being suspicious for various reasons. Nelson & Foltz(49) have also shown that aseptically drawn milk containing above 500,000 leucocytes per ml. causes a colour change in resazurin in 1 hr., but this could not be correlated with the presence of streptococci in incubated samples.

Moir(50) has described the testing of cream with resazurin. He uses 0·4 ml. of 0·05% solution to 10 ml. cream, but the time and temperature of the test are not precisely defined. Jenkins(51) has also studied the applicability of the test to pasteurized cream. He used the normal concentration (0·1 ml. of 0·05% dye to 10 ml. cream) at 37° C. and found that when the cream required over 6 hr. to reduce the dye to a 'goodly pink' it could be considered satisfactory. Only 3% of such samples had a count of over 40,000, and not one caused a customer's complaint. Chilson & Collins(52) report similar results.

Reports of individual experiences with the test have also been published by Brannon(53), who found that the test was more sensitive than methylene blue to organisms causing pin-point colonies.

Thornton, McClure & Sandin(54) have criticized the applicability of the resazurin test to milk on the basis of E_h measurements. Their conditions are not comparable, however, as they used 1 in 20,000 resazurin and 1 in 300,000 methylene blue. They comment on the strong poising effect of the red → white reaction, which is not surprising in view of the concentration of resazurin used (ten times that of the test). They confirm that the first stage is irreversible, all attempts to reoxidize the pink form failing. The blue-red reaction was found to be 'electropositive' and the red-white reaction electronegative to the methylene blue-methylene white reaction. Johns & Howson(55) have also studied this aspect and shown that resazurin is reduced to pink in about three-quarters of the time required to reduce methylene blue to the white stage. Gunderson & Templeton(56) have commented on the precipitate formed when carbon dioxide is added to a resazurin solution. They appear to be unaware that precipitation takes place whenever an acid is added to a solution of a soluble sodium salt of an insoluble acid, and also that resazurin is both a *p*H and an E_h indicator.

Davis(57), Nelson(58) and Thomas(59) have all commented on the unsuitability of some brands of dye and emphasize the need for standardization. One serious objection to the test has been the subjective determination of the end-point. A comparator is now available(60) and its use is recommended. Davis & Twigg(61) have investigated a number of samples of resazurin from different sources with a view to setting up a standard. All samples which behaved normally in milk were found to have the same chemical analysis which, allowing for ash and moisture, agreed closely with the theoretical value for resazurin, $C_{12}H_7NO_4$. Other samples behaved abnormally, gave different analyses, and appeared to be mixtures of resazurin and resorufin, or decomposed and impure material. The usual commercial resazurin is a sodium salt. The insoluble pure resazurin obtained by acid precipitation contains, weight for weight, more dye, so that a 0·05% solution of the acid form is considerably stronger tinctorially than a similar solution of the sodium salt. Neither compositional analysis nor M.P. appears to be of use for standardization, which is most conveniently done in practice with the comparator and with a standard product. Small differences in concentration are of no significance in the test.

The steadily growing interest in the resazurin test has inspired some interesting modifications of the test. These have not yet, however, found general acceptance, and it is not possible to give a satisfactory assessment of their value. One of the earliest was an attempt to use the rapid response of resazurin to bacteria to devise

an ultra-short test giving a result in 5 min. at 45° C.(62). This has now been modified to a 10 min. test at 37° C.(63), which has increased the usefulness, accuracy and sensitivity of the test. The normal concentration of dye (0·0005% in milk) is used now that a comparator is available. The 10 min. test detects most milks reducing methylene blue in less than 2 hr. and is highly accurate in picking out really dirty milks (those reducing in ½ hr.). One advantage of this quick resazurin test over other rejection tests is that a gradation is possible.

Another modification is the resazurin-rennet test(64). This was designed to replace the tests normally used for routine assessment of bacterial quality and degree of mastitis. The only requirement is the addition of *c.* 0·15% rennet to the bench (0·005%) solution and the recording of the clotting times up to 1½ or 2 hr. The strength of the rennet is adjusted to clot normal milk in *c.* ¾ hr. Linked with this test is the 'index of mastitis'(65) which has been devised to enable milk buyers to assess the severity of mastitis in their producers' milk.

Davis & Watson(66) have shown that the resazurin test is three times slower at 18 than at 37° C. The former temperature is easily maintained in a laboratory sink, so that routine testing can be carried out without a thermostatically controlled bath at 37° C. They also report that samples stored at room or refrigerator temperatures reduce relatively faster at 18 than at 37° C.

Schacht & Nichols(67) have investigated the value of different tests for milk quality. Only a small fraction of unsatisfactory milks was detected by odour. Of 730 samples tested, 3% reduced methylene blue in 2 hr., 7% gave a microscopic count of over 100,000, 19% produced a change in colour in resazurin in 1 hr., and 36% were considered unsatisfactory by the resazurin-rennet test. (These standards are, of course, not comparable.) In order to find out whether the last was unjustly condemning good milks, sixty-one farms were very thoroughly inspected and the results correlated with the results of the four tests mentioned. Total agreement was obtained in 38% of cases with the methylene-blue test, 44% with the direct count, 64% with the resazurin test and 85% with the resazurin-rennet test.

Davis(68) has discussed the common tests in use in creamery laboratories and emphasized the unreliability of the usual tests for rejection. The following scheme has been suggested(69). The milk is smelled and a *p*H estimation made by glass electrode or colorimetric test. If the milk passes or fails both tests it is passed or rejected, a *p*H limit of 6·4 being suggested. In the event of contradictory results the milk is put on one side for a rapid resazurin test (10 min. at 37° C.—disk no. 3 or less rejecting the milk).

A review of work on the resazurin test has been published(70).

Methods for coliform organisms

Farrell(71) has compared a number of the usual media for detecting coliform organisms in milk and concludes that brilliant green lactose with 2½ times the normal concentration of dye, and fuchsin lactose broth with seven times the concentration, give fewer false presumptive results than other media. Bartram & Black(72) have also compared a number of liquid and solid media and found that the best of the liquid media for growth purposes in the presence of milk was methylene blue-brom cresol purple and, of the solid media, neutral red bile. The Eijkmann and trypaflavine media were unsatisfactory. Standard lactose broth was superior to all the other liquid media in the absence of milk, and neutral-red bile and violet-red bile agar gave higher counts than standard agar with milk added. Piraux(73) has devised a technique for the 'colorimetric' control of pasteurized milk which involves the addition of trypaflavine, yeast extract and neutral red to milk, covering with a layer of paraffin, and incubating at 37° C. for 24–48 hr. A positive reaction is revealed by coagulation,

gas development and reduction of neutral red. Smit, Krol & van Wijk(74) report that McConkey broth and a medium closely resembling Endo agar gave very similar results. A considerable number of their isolations were considered to be true *Bact. coli* which had lost the ability to produce indole.

Ciani(75) has investigated the methylene-blue test as a possible method of showing the presence of coliforms and concludes that it is not reliable as an indicator of types or numbers.

General

In addition to the five recognized methods already discussed, there are a number of crude miscellaneous tests which attract occasional attention, and Hussman(76) has made rather obvious criticisms of the old fermentation test. No subject in dairy science has been more misunderstood than the acidity of milk, and no test has been more misused than the titratable acidity test. Barkworth(77) has published a useful survey of the literature and recommends that the amount of indicator originally suggested by Soxhlet in 1887 be used (80 mg. %). There is no good reason for suggesting that the quantity of indicator to be added should be enough to give an end-point at *p*H 8·3, since there is no fundamentally determinable end-point for an indicator, this being entirely a matter of visual perception although, of course, loosely related to its *pK*. What is important is to add sufficient to give a sharp end-point and reduce errors due to fluctuations in the measurement of the indicator to an insignificant amount. Davis & Sadek(78) have found that it is necessary to add 4 ml. of 0·5% phenolphthalein to 10 ml. milk to reach that point at which increase in indicator has no effect. They recommend 1 ml. of 0·5% for routine work, which is a convenient amount and sufficiently accurate for the purpose. An exhaustive review of acidity measurements dealing with *p*H and titratable acidity has been published by Døvle(79). This article is valuable for some critical work on the quinhydrone electrode.

In Germany, the alizarol test has been standardized against a colour chart, and Schwarz & Hagemann(80) have shown that faulty results were due to impure alizarin, denatured alcohol or a wrong strength. Davis(81) has tested a large number of indicators, singly and in combination, for their suitability in milk over the important range *p*H 6·4–6·6. He concludes that no single indicator or combination is superior to alizarin. Since this is water-insoluble its use entails the combined alcohol test, which does not meet with approval in all laboratories. It is hoped that a Tintometer disk for alizarin will be available shortly.

Graf(82) has described a new fluorescent staining technique for bacteria and cells, and Behmer(83) a modified methylene blue-fuchsin stain for cells in milk.

Davis(84) has published a general article on flavours in milk.

Milk control

Lochhead(85) has issued a popular bulletin on the production of clean milk which gives some useful figures for the relative effect of various factors in controlling the bacterial count of the milk. Thomas & Jenkins(86), in common with other investigators, find that the quality of designated milk in England and Wales has deteriorated in the last few years, although these milks are much superior to undesignated milks. Although fewer samples of pasteurized milk now fail to pass the phosphatase test, the proportion of samples not attaining the bacteriological standard has not fallen. In America, Babcock has issued a report of the American Public Health Association Committee on Milk and Dairy Products(87) which gives figures for proportions of milk pasteurized in towns of various sizes and the incidence of outbreaks of milk-borne diseases.

Little is known about the bacteriological aspects of human milk. Sauer(88) has tried the effect on the bacterial content of heating human milk to different temperatures.

The effect of vacuum sealing of milk bottles has been investigated in New Jersey(89), where it has been found that this method slightly delayed bacterial growth but not increase in acidity.

Trebler(90) has given a detailed specification of a new type of milk churn which drains satisfactorily and is easier to clean.

II. LACTIC ACID AND ALLIED FERMENTATIONS

(*a*) Bacterial metabolism

General. Little work appears to have been done on physical conditions in relation to bacterial growth. Rahn & Bigwood(1) consider that the death of cells held at 0° C. is due to the toxic action of oxygen in the absence of growth mechanisms which would otherwise have consumed it, as viability was increased by removal of oxygen. Dorn & Rahn(2) have measured the optimum temperature for rate of growth, final number of cells and final concentration of fermentation products. They report 34° C. for *Str. lactis* and 38–39° C. for *Str. thermophilus* when the rate of multiplication is taken as a criterion, and 40 and 47° C. respectively when the rate of fermentation was measured. From 2·5 to 10 times as much lactose was required for the doubling of one cell (reproductive efficiency) at high temperatures as compared with low temperatures.

Davis & Thiel(3) have studied the important factor of *p*H in relation to growth and gas production by streptococci and lactobacilli. Practically all streptococci in their groups I and II grew at *p*H 8·8 but only the enterococci at *p*H 4·8. All the heterofermentative (group III) types grew at *p*H 4·4. *Str. cremoris* could be differentiated from *Str. lactis* by failure to grow at *p*H 9·2, and *Str. lactis* from *Str. faecalis* by failure to grow at *p*H 9·6. Very few lactobacilli grew at *p*H 8·8, but most of those tested grew at *p*H 4 under otherwise favourable conditions. Fornachon, Douglas & Vaughn(4) report that heterofermentative lactobacilli from wine fermented sugars at *p*H 5·5 but not at 6·8. Baumgartner & Knock(5) have discovered that each of the organisms studied had a *p*H optimum that was the same for resistance to heat and alcohol and for stability of suspension. It is considered that the state of hydration of the bacterial protein is the factor responsible for this phenomenon. In their studies of conditions determining stationary populations and growth rates for *Bact. aerogenes* Lodge & Hinshelwood(6) have found that the final stationary population was independent of the size of the inoculum, so that the hypothesis that adverse conditions result in cessation of growth at the *n*th generation cannot be accepted. Stationary population-*p*H curves showed sharp inflexions, suggesting that it was a labile intermediary rather than the amphoteric protein which was affected by *p*H. They found the mean generation time to be at a minimum at 40° C. and the stationary population at a maximum at 25–30° C. Not only growth but also the lag period were dependent upon the presence of magnesium. If added after a certain interval the element had no effect. Rahn(7) has shown that bacteria need CO_2 not only during the lag phase but during the period of logarithmic growth.

Fermentation. While work on the mechanism of the lactic-acid fermentation has continued, chief interest in the field of carbohydrate metabolism research has centred on butter aroma (diacetyl) formation.

Werkman(8) has written a review of recent work on the bacterial dissimilation of carbohydrates, and Davis(9) has shown by a study of the Pasteur reaction in lactic-acid bacteria that these bacteria fall into three well-defined groups, one with equal aerobic and anaerobic fermentation, a second with the usual aerobic depression of

fermentation, and a third in which a greater fermentation is found in the presence of air. Smith & Sherman(10) have studied the conversion of sugar by washed cells of streptococci and found that about 90% was converted to lactic acid. Friedemann(11) has also investigated this aspect under rather different conditions and found that the *viridans* group produced slightly less lactic acid than the lactic or haemolytic streptococci. Two types of metabolism were found, one producing 2 mol. of CO_2 and 2 mol. of alcohol, and the other 2 mol. of formic acid and 1 mol. each of acetic acid and alcohol. Nelson & Werkman(12) in their studies of the fermentation of fructose by heterofermentative lactic acid bacteria found that with both *L. lycopersici* and *Leuc. dextranicus* similar breakdown mechanisms were involved and were essentially the same as those for dextrose. Iwasaki(13) has also studied pentose-fermenting lactobacilli.

Davis(14) has summarized the sources of error in the ordinary cultural methods for the determination of bacterial sugar fermentations and suggested a metabolic method. This consists in incubating cell suspensions in phosphate buffer at *p*H 6·8 with brom-cresol purple as an indicator and adding the sugars with a scoop. After 16–24 hr. incubation at 37° C. the tubes are observed. The method is simple, rapid and of high reproducibility, and has additional advantages in the low proportion of doubtful reactions and the fact that aseptic precautions are unnecessary. Robbins & Lewis(15) have studied the fermentation of mono- and di-carboxylic sugar acids by twenty-four types of bacteria. They suggest that fermentation of *d*-mannonic acid by *Str. lactis* and *Str. liquefaciens* would be useful in differentiating these bacteria from *Str. agalactiae* which does not ferment it. Thiel has made a detailed study of the influence of various factors on the fermentation end-products of the heterofermentative streptococci(16) and lactobacilli(17). Temperature, oxygen tension, acidity and presence of growth factors (yeast autolysate) were the variables studied. Broadly speaking, relatively more lactic acid is produced under anaerobic conditions and in the presence of chalk, and relatively less acetic acid under anaerobic conditions, at low temperatures, and in the presence of yeast autolysate and chalk, but more alcohol is produced under these four conditions. *Str. citrovorus* was able to hydrolyse residual lactose, and the respiration of the heterofermentative streptococci failed to suppress aerobic fermentation. Very similar results were obtained with the lactobacilli. It is suggested that estimation of alcohol and CO_2 is of more value in classifying these bacteria than the determination of acetic acid. In this connexion it is of interest to note that Stone(18) reports that heterofermentative lactobacilli produce less by-products (CO_2, acetic acid and alcohol) in the presence of glutathione. These organisms apparently contain more glutathione than the homofermentative lactobacilli. Abbot(19), on the other hand, found that tomato juice markedly increased the yield of aroma substances in butter cultures. Citric acid was not the responsible substance. Moore, Dimler & Link(20) have described a new method for estimating the proportion of *d*- and *l*-isomers in lactic acid. The acid is condensed with *o*-phenylenediamine to form 2 (α-hydroxyethyl) benzimidazole which is then precipitated as the silver salt, the weight of which, and the rotation of the benzimidazole (which is high), give the required information. Katagiri & Murakami(21,22) have continued their work on the optical aspects of lactic-acid fermentation. They found that a *l*-acid producer decomposed (+) phosphoglyceric acid, a *d*-acid producer the (−) form, and a *dl*-acid producer all forms. Their racemiase racemized 2- and 3-phosphoglyceric acid.

All work on diacetyl production naturally depends on methods for estimating diacetyl and acetoin, and Cox & Wiley(23) have studied Pien's method (*p*-diaminobenzidine condensation) and increased its accuracy to give a reasonably reliable and quick method for small amounts of the substance. Komm & Flügel(24) have described a colorimetric method based on condensation with *m*-dinitrobenzoic acid in alkaline solution, and claim a satisfactory accuracy using diacetyl standards of known

strength. Mohr & Arbes(25) have also tried the quick method of Schnalfuss & Werner, and report favourably on it. They made the interesting observation that small amounts of iron, copper and manganese caused an increase in the production of diacetyl, especially with skim milk, and that butter-washing water contained significant amounts of iron and manganese. Tests 48 hr. after washing with water containing similar amounts of metal salts showed that no effect could be detected, but addition of the salts to cream before, but not after, souring caused a rise in diacetyl content. Working with very weak solutions of H_2O_2 produced a marked rise in diacetyl.

Brewer(26) has studied the chemical mechanism of diacetyl formation and found that *Str. paracitrovorus* only attacks citric acid in the presence of sugar. This organism formed only lactic acid, ethyl alcohol and CO_2 from sugar, but in the presence of citric acid produced also acetoin, 2–3-butylene glycol and acetic acid. Sometimes pyruvic acid could be detected as an intermediate compound. Other hydrogen donators could not replace sugar and the reaction was not inhibited by arsenite. Hammer & Werkman(27) have also reported that *Str. paracitrovorus* does not attack citric acid in the absence of sugar. A dissimilation scheme for citric acid has been presented by van Beynum & Pette(28). Virtanen, Kontio & Storgårds(29) do not accept that citric acid is the source of aroma compounds in cream souring, but claim that they are formed by sugar fermentation, as with *Bact. aerogenes*. They were unable to obtain acetoin from citric acid. Further evidence for the citric-acid theory, however, is derived from the work of Slade & Werkman(30) on cell suspensions of *Str. paracitrovorus*. In the presence of lactate, citrate is broken down to oxaloacetic and pyruvic acids, the latter being converted either to lactic acid or to acetic and formic acids. Fluoride has some, and pyrophosphate no inhibitory effect. Azide, arsenite and iodoacetic acid, however, are strong inhibitors. Storgårds(31) has also studied the reaction with washed cells and found that the presence of calcium or barium was necessary. This worker considers that acetoin arises from the sugar via pyruvic acid, and that citric acid merely plays the role of hydrogen acceptor. Using an enzyme preparation from *Bact. aerogenes*, Silverman & Werkman(32) found that phosphate, cocarboxylase, magnesium or manganese, and an acid *p*H were necessary for the production of acetoin. It is evident that further work is required before the question of the source of diacetyl can be decided upon.

Turning now to the practical side, and particularly the factors controlling aroma production in practice, we may consider the work of Wiley, Cox & Whitehead(33) on the relative value of single and mixed strains of *Str. cremoris*. Working at 7° C. single strains produced as much diacetyl in 18 hr. as mixed cultures containing betacocci. Their results suggest a quite different mechanism at low temperatures, so that the behaviour of a culture at 21° C. is no guide if low-temperature ripening is used. More aroma was obtained in aerated pasteurized milk than in steamed milk. In a further paper(34) they report a faster production at 21 than at 7° C., but after 24 hr. a higher total at 7 than at 21° C. when single strains of *Str. cremoris* were used. Mixed cultures containing betacocci gave similar results at 7° C., but at 21° C. a rapid production in the first 12 hr. was followed by rapid disappearance of the diacetyl, and in 40 hr. there was less in the 21° C. culture than in the one at 7° C. Betacocci alone in milk gave no diacetyl, but when the milk was acidified to *p*H 4·2 a similar phenomenon was observed. Unlike *Str. cremoris*, the betacocci cannot only produce but also destroy diacetyl. Prill & Hammer(35) have also studied diacetyl production in butter cultures. Their data suggest that citric acid and aeration are important factors. Increased yields could be obtained by using low temperatures and addition of citric acid. The possibility that diacetyl may be destroyed by other organisms should not be overlooked in dairy practice. Virtanen & Kontio(36) have shown that *B. punctatum* could destroy some 90% of both acetoin and diacetyl, while *B. vulgatus* destroyed

as much acetoin but only 40–60% of the diacetyl. Other types destroyed varying amounts of each substance. Betacocci are not the only organisms producing appreciable amounts of aroma substances. An organism culturally resembling *Str. lactis* but producing large amounts of diacetyl has been described under different names, e.g. *Str. diaceti-lactis* and *Str. citrophilus*. In India, Karnad(37) has described *Str. lactis aromaticus* which appears to be a closely related organism. Baba(38) has classified thirty types of mannitol-forming bacteria isolated from fruit, cereals and dairy products, and Sakaguchi & Tada(39) have described *Bact. succinicum* which forms large amounts of succinic acid from various organic acids and appears to be of the *Bact. coli* type.

Growth factor requirements. Considerable interest in the growth-factor requirements of lactic-acid bacteria has been displayed over the period of the review, not only because of its intrinsic interest and importance but also because of the development of microbiological methods of assay for riboflavin and pantothenic acid.

Möller(40) considers that the casein hydrolysate used as a source of amino acids in media for lactic-acid bacteria contains three growth factors, *F* (possibly pantothenic acid), *G* and *H*, the last possibly consisting of biotin, nicotinic acid and an unknown factor *H*. The bacteria could apparently synthesize cozymase from nicotinic acid and adenine. *L. casei* but not *L. plantarum* required a factor present in brewers' yeast and the liver of young animals. Snell, Strong & Peterson(41) now report that the liver factor previously described by them is pantothenic acid. *L. arabinosus* and *L. casei* were stimulated by nicotinic acid. Other organisms were not sensitive to these substances but responded to yeast, liver and malt but not to other listed substances. Snell & Peterson(42, 43) have shown that hydrolysed casein, riboflavin, pantothenic and nicotinic acids do not constitute a complete medium for *L. casei*, but require supplementing with a factor in yeast and liver. Norite adsorption differentiated two constituents in this factor. Wood, Geiger & Werkman(44) have studied the requirements of the heterofermentative lactobacilli which appeared to be inorganic salts, amino acids, aneurin, riboflavin and an ether extract of yeast, the last not being replaceable by recognized substances. Aneurin and riboflavin both stimulated growth but neither was essential in the presence of the other. The amino acids could be classed in six groups: essential, basic, dicarboxylic, ring compounds other than tryptophane, simple, and leucine. Omission of any one group resulted in inferior growth. Bohonos, Hutchings & Peterson(45) have reported that B_6 is essential for the high-temperature lactobacilli but not for the low-temperature heterofermentative organisms, which appear to be capable of synthesis. Krauskopf, Snell & McCoy(46) have surveyed the pantothenic and riboflavin requirements of bacteria. Lactic- and propionic-acid bacteria require the former, and riboflavin sometimes stimulates growth of lactic-acid bacteria. Other organisms are variable in their requirements. Snell & Mitchell(47) investigated the effect of various pyruvic and pyrimidine bases by adding them to a basic medium containing casein hydrolysate, acetate, glucose, cystine, tryptophane, aneurin, riboflavin, pantothenic acid, vitamin B_6, nicotinic acid, biotin and salts. Different substances stimulated different types, and it was found that cytosine could replace uracil and xanthine guanine. The new growth factor required by *L. casei* and described by Snell & Peterson has been shown by Stokstad(48) to behave like a dinucleotide containing guanine but not adenine. A mixture of guanine and thymine was of some potency in replacing it.

Orla Jensen & Snog-Kjaer(49) have compared a number of plant extracts and other materials for their growth-promoting value. The authors concluded that riboflavine rather than bios was the chief factor, and showed that the stimulation was enhanced by amino acids, etc., in the extract, the sulphur-containing substances being important for some types. Milk contains two thermostable substances inhibitory to the lactic-acid bacteria. Virtanen, Karström & Kahra(50) have found that heated

whey and yeast extract, but not lactose, whey ash or aneurin stimulated *L. helveticus* in phosphate buffer. Davis(51) studied the effect of several plant extracts and yeast autolysates on acid production by lactic-acid bacteria in milk. Usually yeast autolysate was best, but with the heterofermentative types beer wort was most active so that this appears to contain some substance essential to these organisms and not present in yeast autolysate. Some sugars but not citrate accelerated acid fermentation. Vitamins B_1, B_2 and cocarboxylase were without effect. Pan, Johnson, McCoy & Peterson(52) similarly report that unheated malt sprouts accelerated fermentation by *L. Delbrucki*. Of interest from the point of view of cheese ripening is the observation by Hansen(53) that autoclaved streptococci increased the final acidity of *L. casei* in milk.

Snell & Strong(54) have introduced an assay for riboflavin based on stimulation of acid production by *L. helveticus*. This has now been adopted as an official method in America(55) and has been used by Arnold, Lipsius & Grebe(56) for estimating riboflavin in dried milk, etc., and the authors claim to have obtained reliable results. Davis & Newland(57) have investigated the Snell & Strong method. Tests on numerous strains of lactic-acid bacteria showed that there was no relation between type or species and riboflavin or pantothenic-acid requirement. In general, the latter appeared to be more important for lactic-acid bacteria. To secure satisfactory blank values, one-tenth the inoculum was necessary, and milk consistently gave high values for riboflavin, suggesting the presence of other growth factors. Pennington, Snell & Williams(58) and Strong, Feeney & Earle(59) have adapted the method for the assay of pantothenic acid. Feeney & Strong(60) find that the ordinary technique for the microbiological assay of pantothenic acid is unsatisfactory as the recommended medium is deficient. The addition of carbon-treated yeast extract gave better growth and a better assay. Either asparagine or glutamine could replace this addition to some extent. Some light on the possible mechanism of stimulation by certain extracts is afforded by the observation of Anderson(61) that manganese (0·04 p.p.m.) stimulated the growth of lactobacilli. Cohen, Snyder & Mueller(62) have studied the growth-factor requirements of *C. diphtheriae*.

Media. Britain is much behind others in standardizing microbiological methods for dairy products. A start has been made by the publication of tentative British Standard Methods for the examination of butter(63), which cover coli, yeast and mould, tributyrin- and casein-splitting types and saccharolytic organisms. Sugar fermentations are frequently used in dairy bacteriology for classification purposes, but indefinite results are common, due, amongst other factors, to partial decomposition in sterilizing. Davis & Rogers(64) have studied the extent of decomposition of sugars sterilized by different heating methods and have emphasized the importance of keeping the *p*H of sugar media below 7. They recommend 'momentary autoclaving' as being only slightly more destructive than intermittent steaming and more reliable. Gelatine media containing heat-resistant spores can be sterilized in this way. Kass & Palmer(65) have similarly studied the browning of autoclaved milk, which is associated with acid production, fall in optical activity and 'copper-reducing power', and formation of ketoses. The theory put forward is that of caramelization of lactose by the casein and adsorption of the brown material by colloidal caseinates. Leifson(66) has studied the preparation of peptones from casein by peptic, papainase and tryptic digestion, and reports that the last method gives the best peptone. Nelson & Foltz(67) recommend tryptone glucose skim-milk agar with 5% skim milk for the determination of proteolytic organisms in dairy products. The value of tributyrin agar has been explored by Wolf(68), who obtained highest counts at 28° C. and confirmed the toxic nature of tributyrin. Higher counts were obtained with 0·5 than with 1% substrate, and tributyrin agar gave more zones than butter-fat agar. Streptococci and lactobacilli are able to hydrolyse tributyrin.

(*b*) Lactic-acid bacteria

Frost & Engelbrecht(69) have published a monograph on streptococci in milk with particular reference to disease, which cannot be adequately reviewed here, but which makes a very useful, if unorthodox, addition to the literature especially for research workers. Kitahara(70) has described a classification which takes into consideration racemiase production in addition to well-accepted properties. Although interesting and describing some apparently new types, this classification is likely to confuse the situation by introducing catalase-positive types. Davis & Rogers(71, 72) have grouped lactic-acid bacteria into three groups on the basis of respiration and fermentation mechanisms: (i) homofermentative, mesophilic, saprophytic, non- (or slightly) respiring; (ii) preferring higher temperatures, the streptococci pathogenic or potentially pathogenic—subgroup (*a*) haemolytic, (*b*) 'green-formers' (H_2O_2 producing), (*c*) enterococci (resistant types producing diacetyl) (there are no lactobacilli in subgroup (*a*)); (iii) heterofermentative, mesophilic, high respiration but no H_2O_2 produced.

A vexed question of long standing is the question of the separate existence of *Str. lactis*. Sherman, Smiley & Niven(73) have now produced sera against *Str. lactis* which gave satisfactory precipitation reactions with all strains of *Str. lactis* tested, but not with *Str. faecalis* or any other types tested. Most of the *Str. cremoris* strains tested reacted weakly with these sera. Okulitch(74) has also studied dissociation in *Str. lactis* and *cremoris* and found that transition from *S* to *R* resulted in loss of power to ferment lactose but not to ferment dextrose. Dissociation was erratic and the factors responsible for the change could not be elucidated. Sherman, Chase & Niven(75) have similarly studied variability in group B streptococci and state that haemolysis and the fermentation of salicin and lactose are highly variable. Murphy(76) also reports that 44 haemolytic out of 85 strains of *Str. agalactiae* fermented trehalose, while 18 non-haemolytic strains fermented salicin but not trehalose. Generally speaking salicin fermentation was very variable, and no relation between trehalose fermentation and pathogenicity could be noted.

Davis & Rogers(77) have described a gas-forming and proteolytic variant of *Str. agalactiae*, and an aesculin hippurate broth(78) as a supplementary medium to yeast litmus milk for the cultural diagnosis of this organism. They recommend aesculin ferric chloride yeast litmus milk(79) as the best single medium. The same authors have published an account of a systematic investigation of the reactions of streptococci and lactobacilli in blood agar(80). They consider that all reactions other than true β haemolysis are useless for identification purposes owing to variability, and describe a new reaction, 'bleaching', frequently given by saprophytic types. A simple method of differentiating true β from other types of haemolysis is described. The differentiation of *L. bulgaricus* from *L. acidophilus* is another example of a classical dispute of long standing in bacteriology. Sherman & Hodge(81) have described four simple methods of differentiation. Lazicova(82) has compared a number of strains of *L. acidophilus* from different sources and concluded that they could all be considered within the limits of variability of one species. Harrison(83) was able to classify 101 of 111 strains of lactobacilli isolated from dental caries in two main groups. In the larger one 75 strains clotted and reduced litmus milk and fermented mannitol, and the remaining 26 were only weak acid producers. Kitahara(84) has classified 72 strains of lactic-acid bacteria isolated from dairy products and described a new species, *Bact. caseolyticum*, which produces protein decomposition and a cheesy odour in milk and has a thermal death-point of 80–90° C. (This author's use of the term 'lactic-acid bacteria' is wider than that usually accepted.) Soriano(85) has classified lactobacilli from a fermented maize drink on the basis of milk clotting and gas production in cereal musts, and Kitahara(86) has classified bacteria from

cereal mashes in his system. A general account of the lactic-acid fermentation has been written by Rodenkirchen(87); the same worker(88) has also studied antagonism effects and reported that a cell-free filtrate of a *Str. thermophilus* type inhibited growth of *Str. lactis* and aerobic spore-formers but not of other types. The substance responsible was heat-resistant. Butyric-acid bacteria destroyed riboflavin in milk and inhibited *Thermobacterium* and thermophilic streptococci strongly, *Str. lactis* fairly strongly, and *Streptobacterium* slightly or not at all. Alexandre & Cacchi(89) have investigated factors controlling the antagonism between *B. coli* and *L. bulgaricus*.

Davis(90) has described methods for the maintenance of pure cultures of lactic-acid bacteria, and Sullivan(91) reports that *L. acidophilus* remains viable longer in cooked meat media than in milk. Hammer(92) has published results obtained with methods of drying butter cultures for distribution. Düggeli(93) has written a comprehensive review of the micro-flora and uses of sour-milk products and Dorner(94) has reviewed his researches on the propionic-acid bacteria.

(*c*) Starters

It is held by some workers that the major cause of slowness or complete arrest of acid production is bacteriophage (see p. 366). There is little doubt that many dramatic failures which were not investigated were due to phage, but it is probable that failure to develop proper acidity or otherwise to behave normally, also arises from other causes.

For example, Prouty(95) has shown that mastitis milk with a *p*H value above 6·9 failed to support active growth of *Str. lactis*, and Elliker & Frazier(96) showed that although the growth of *L. helveticus* (Swiss cheese starter) was good in stale sterilized milk reconstituted from skim-milk powder, its heat resistance was much reduced. The addition of 'Neopeptone' restored the original growth properties of this milk, the oxidation-reduction potential of which was found to have risen on storage. Davis(97) found that heated serum, heated blood or unheated blood had little effect on the growth in milk of most species of lactic-acid bacteria. Unheated serum markedly inhibited *Str. lactis* but not *Str. cremoris*. This effect with *Str. lactis* was very evident when inocula were small but was abolished by a 1% inoculum, thus suggesting the unlikelihood of slowness in the cheese vat due to serum infiltration into the milk.

Curran, Evans & Leviton(98) found that milk treated with 1% H_2O_2 at 55° C. for 2 hr., the residual H_2O_2 being destroyed by added catalase, severely inhibited the growth of *Str. lactis* but not *Pseudomonas* and *Bact. coli*. It is interesting to note in passing that raw human milk is strongly bactericidal for streptococci, an effect which is abolished by boiling for 3 min. (Catel(99)). Penicillin, a representative of a large number of bactericidal substances of biological origin, did not, according to Bornstein(100), uniformly inhibit the enterococci and *Str. lactis*.

Tarassuk & Smith(101) found that rancid milk inhibits growth and acid formation of *Str. lactis*, and consider that lowering of the surface tension is responsible.

Harrison & Dearden(102) recorded experiments which suggest that, in some starters, active may be replaced by less active forms. Although no phage could be demonstrated in the slow cheese investigated, they suggested that phaging of the more active strains may have taken place resulting in the establishment of a strain incapable of growth at 40° C. One of their starters was found to have resolved itself into what was apparently a single strain of *Str. lactis*.

In the cultivation of starters it is well to bear in mind that, as with cultures of other organisms, they are susceptible, amongst other things, to environment, and time and temperature at which they are grown result in changes in the lag period when they reach the vat, the power to form acid over the necessary range of tem-

perature and the rapidity of growth, terminating perhaps in unsatisfactory acid formation.

Even with pure cultures of *Str. cremoris*, variations for which the governing conditions could not be defined were noted by Hunter(103). The variants could be distinguished on colony form, one grew much more slowly than the parent strain, and the other failed to ferment lactose. Morphologically there were no differences from the original strain and all were attacked by the same phage. Other substrains were slow or rapid acid producers, were ropy or were resistant to high temperatures. Harrison & Wolf(104), in examining starters from commercial creameries, found that a high proportion of the considerable number examined failed in the first 7 hr. after inoculation to give a proper acidity even though no bacteriophage could be demonstrated. They noted that all these slow starters gave their proper acidity of 0·9–1·1% (as lactic acid) within 24 hr., so that final acidity is by no means a safe criterion of a satisfactory starter. Whitehead, Hunter, Riddet & Sawyer(105) maintain that differences in the cheese made with single-strain starters can be detected, but that satisfactory cheese of true Cheddar type can be manufactured with these starters. Riddet & Whitehead(106), using two single-strain starters have shown that the character of a cheese is mainly determined by the acidity at whey drawing. The rate of acid development and the acidity at milking and salting have small effect. The optimum acidity of whey from the curd after drying varies from 0·20 to 0·28% with the composition of the milk. Acidities which were too low gave weak body and those which were too high gave sticky, firm body. Variations of 0·03% (lactic acid) from the optimum acidity caused these effects. With active starters such as these, cooking times can be safely reduced. Modifications in the process of making were required to suit the particular starter in use.

Dahle & Anderson(107) found that a pure culture of *Str. lactis* used in Cheddar cheese making gave the same rate of development of flavour acidity and soluble protein during ripening as a commercial mixed starter. When *Str. citrovorus* was added, bitterness developed, and when *Lactobacillus bulgaricus* was used a pronounced acid flavour was found. They noticed also that the numbers of organisms in the cheese were greater at 43 than at 63° F. Nilsson(108) has confirmed Barthel's observation that *Str. lactis* in pure culture and in mixed starter can split casein.

Gibshman(109), presumably working with butter cultures, showed that mixed cultures of *Str. lactis*, *Str. cremoris* and *Str. citrovorus* gave better results at 20–22 than at 30° C., as the too rapid growth of vigorous acid-formers (*Str. lactis*) affected the production of volatile acids, acetoin and esters. Long culture at 20–22° C. did not affect the characteristics of the starters.

Drying in vacuo at low temperatures has been used in recent years for the preservation of bacterial cultures over long periods. Starters in powder form have for many years been in common use, but they have certain disadvantages. If single-strain cultures are to be maintained separately and then mixed in bulk starters for use in the vat, it will mean that creameries will have to be in possession of reserve cultures which must, on subculture, reach full activity quickly. Mattick & Rogers(110) have explored the possibilities of using the freeze-drying process with *Str. cremoris* and *Str. thermophilus*, selected because of their comparative delicacy. Organisms from cultures grown for 24 hr. in a suitable medium were separated by centrifuging, suspended in broth + 5% cow serum, dried, frozen in vacuo, sealed in ampoules and kept at room temperature in the dark. *Str. cremoris* was fully vigorous after 11 months. *Str. thermophilus* was still recoverable but was of reduced vigour.

Hammer(111) has made similar tentative experiments with butter cultures, and it is clear that further work to define the optimum conditions of drying is needed.

The earlier work of Whitehead and his colleagues in New Zealand on the occurrence and effect of bacteriophage, active against single-strain starters, during cheese

making is now well known, but recent developments are of considerable importance. It will be recalled(112) that they proposed an empirical and temporary solution to the problem of slow working by increasing the inoculum of starter to a quantity which is more comparable with that used in England. (The latter may partially account for the comparative rarity of complete 'pack up' here.) They also advocated a strictly aseptic technique during starter preparation. They suggested that the development of phage was stimulated by aeration, but repeated and sustained attempts to confirm this in our own laboratory failed completely and a more probable explanation (dealt with later) is now offered by the New Zealand workers. Since the last review workers in other parts of the world have found phages active during cheese making (104, 113, 114, 115), not only against single-strain starters as used in New Zealand but in mixed strain starters. What constitutes a *strain* is not defined. Whitehead & Hunter(116) have shown that there is some, if not complete, specificity of the phages active against *Str. cremoris*; which may account for the facts, generally conceded, that mixed strain starters are less liable to complete failure than single strains, especially in pasteurized milk. Whitehead & Hunter also found that some phages act more rapidly and completely at 37 than at 30° C.

Forms of streptococci resistant to phage were easily found by continuing the incubation of lysed cultures. These forms may be permanently or only temporarily resistant, and other phages active against the resistant strains (but not the original) were found when they were used as starters. They finally concluded that phage relationships do not conform to any simple rule.

Meanwell(115) suggests that the partial failure of acid production when a mixture of two streptococcal strains is grown in the presence of a phage active against only one of them may be another example of the action of 'nascent' phage described by Evans(117) for group B streptococci.

This author holds, with others, that in the present state of knowledge it is preferable not to risk serious 'pack up' by using single-strain starters (in spite of their manifest practical advantages) but to use mixtures of strains so that with the necessary precautions the likelihood of complete or even serious partial failure may be lessened.

Meanwell(115) and Harrison & Wolf(104) use methods of titration after the addition of suspected whey to a milk culture of starter as a means of demonstrating slowness. The latter authors draw attention to the fact that the final acidity of a starter is no guide to its probable behaviour in the vat, since they found that many starters which failed to form appreciable acidity within the first 7 hr. after inoculation gave quite normal acidities when retitrated after overnight incubation. They also encountered many sluggish starters which failed to yield bacteriophage against any of the strains isolated.

Whitehead and his co-workers reported some years ago their belief that aeration of cultures sometimes resulted in the spontaneous appearance of phage. In a more recent paper(118) they have revised their opinion and have shown clearly that phage particles occur in the air of cheese factories, especially where fine spray, originating from whey separators, is present. Using the normal bacteriological technique, it was found to be virtually impossible to avoid infection, but the erection of a small separate building and the adoption of a rigid technique appears to have gone far towards solving the problem. It is interesting to note that Whitehead & Hunter(118) found that cotton-wool plugs arrested phage particles. Hunter & Whitehead(119), in a search for chemical substances for the destruction of phage, tried $KMnO_4$, H_2O_2, HCHO and $HgCl_2$ and found that the effects were descending in the order given. Hypochlorite at a final concentration 0·05 % available chlorine destroyed eight strains of phage in less than 1 min. It therefore appears that hypochlorite may be useful in helping to control air-borne phage.

Finally, Davis(120) has compiled a review of slow cheese problems discussing bacterial nutrition factors, inhibitors of divers origins, bacteriophage and other problems.

(*d*) Cheese

The use of pasteurized milk for cheese making has provided a means of controlling many of the faults common to cheese made from raw milk. Although this has increased the chances of producing cheese of uniformly good quality, the cheese so made ripen slowly and even when mature lack much of the flavour characteristic of the type of cheese. Modifications in technique will probably eventually restore and retain these characteristic flavours, but in order to establish the correct technique a deep-seated knowledge of the chemical and biological processes that bring about the desired reactions is essential. The addition of the necessary bacteria and enzymes would go far towards establishing the desired reactions, but linked with the biological factors are other chemical and physical factors which must also be taken into consideration.

Mycological factors. During the period under review much has been done to increase our knowledge of the characteristic flora at each stage of the manufacture and ripening of several types of cheese. The succession of changes in the counts of various groups of bacteria during the ripening of Cheddar cheese, raw and pasteurized, has been followed by Mattick & Hiscox(121) and Dahle, Anderson & Triebold(122) using selective media, but no reports are given of detailed study of species. The lactobacilli have been studied in some detail by Sherwood(123,124,125,126), who classified 720 strains isolated from New Zealand Cheddar cheese in six broad groups. Of the bacteria isolated from pasteurized milk cheese 62% were *Sbm. plantarum* (four varieties), 16% *Sbm. casei*, and 12% were intermediate (*plantarum-casei*). Two of the four varieties of *Sbm. plantarum* produced objectionable flavours. Heavy inoculation of pasteurized milk with *Sbm. plantarum* and *Sbm. casei* resulted in a deterioration in flavour of the cheese. Good results were obtained, however, when the inoculum was just sufficient to give a number of the same order as that in ordinary commercial milk. So high a proportion of lactobacilli survive the usual New Zealand method of pasteurization of cheese milk that a significant amount of inoculum is required only when dealing with a clean milk supply. Control of flavour by this method will increase in value as the quality of milk improves. The raw-milk supplies were the probable source of gas-producing lactic-acid bacteria (*L. brevis* and less frequently *L. plantarum* and strains of *Betacoccus*) shown to be the cause of 'slit-openness' in Cheddar cheese. The lactobacilli gain access to the raw milk from dairy utensils, such as milking machines, and from farmyard dust. Lane & Hammer(127) found that the smear-culture technique gave larger and more numerous colonies of the chromogenic and proteolytic types of bacteria in Cheddar cheese, but poured plates were more satisfactory for the lactic-acid bacteria. Harris & Hammer (128) tried the effect on the flavour of Cheddar cheese of the addition of various bacteria to the pasteurized milk from which it is made. Of thirty-four strains of micrococci only thirteen improved the flavour, seven, in fact, caused undesirable flavours. Large numbers of propionic-acid bacteria (small numbers of some strains) and small quantities of calcium propionate improved the flavour, but too much of the salt gave a flavour of Swiss cheese. The effect of individual strains varied, hence the selection of cultures for inoculation into cheese milk should be on the basis of strain and not species.

The addition of large numbers of propionic bacteria improved the flavour of Swiss cheese, but appeared to have no influence on the eye formation (Babel & Hammer(129)). Propionic-acid bacteria were found by Demeter & Janoschek(130) in all the types of cheese examined by them. Usually the numbers were very small, but in Emmental and Iowa Blue cheese they were high both in actual figures and in

relation to the lactic-acid bacteria. At first sight the latter seems a rather surprising result, but in a subsequent paper giving details of their experiments(131) the authors reveal that the Iowa Blue cheese was a Gouda type and not the Roquefort type normally associated with this name. Janoschek(132) traced the rise and fall of the numbers of lactic- and propionic-acid bacteria during the manufacture and ripening of Emmental cheese. The former reached a maximum in the press (about 24 hr.), the latter not until the 70th day, when they greatly outnumbered the lactic-acid bacteria. Dorner & Mosimann(133) found large numbers of propionic-acid bacteria in cheese from the press if cultures of propionic-acid bacteria had been added to the cheese milk. Cheese from some factories not using cultures contained very few at this stage. Propionic-acid cocci were rarely found in the cheese, although they were eight times as numerous as the rod forms in the cheese vats. In ripe Hungarian Emmental cheese 89% of the bacteria were acid producers. The various types of bacteria were studied in further detail by Ary(134). Aerobic spore-forming bacteria, the apparent cause of bitterness in Swiss cheese, were inhibited by the use of an active starter, cooking at temperatures above 53° C. and heavy salting (Burkey, Rogosa & Farrar(135)). Farrar(136) has reported an improvement in the quality of factory-made Swiss cheese through the introduction of pure-culture starters and better methods of starter propagation. Lost heat tolerance in cultures of *Str. thermophilus* and *L. helveticus* could be restored by prolonged cultivation under temperature-shocking conditions (Burkey & Rogosa(137)). The activity of a starter is favourably influenced by the use of proper temperatures and times for heating the milk used in the preparation of the bulk starter (Tyler & Weiser(138)). Peppler & Frazier(139) found that active cultures of *L. helveticus* and *Str. thermophilus* could be prepared with considerable latitude in time and temperature of incubation. The resistance of the organisms was high when grown at 37° C. in a good medium such as reconstituted milk(96), but the resistance was enhanced by fortification of the medium with neopeptone or malt extract. Starters of great resistance were produced by improving the medium and incubating at 40 instead of 37° C. This may be an advantage when cooking temperatures are higher than usual to avoid gas formation in the cheese.

Foster, Garey & Frazier(140,141,142,143) have followed the changes in the bacterial flora of Brick cheese from the time of manufacture. *Str. lactis* did not grow in the vats when the cooking temperature was 104–112° F., and rapid growth did not begin until some hours after dipping, but very high numbers were reached within 24 hr. of this time. *Str. thermophilus* began to grow rapidly in the vat during cooking at 104° F., but the rate decreased as the curd cooled after dipping. Maximum numbers were reached within 12 hr. of dipping. A mixture of *Str. lactis* and *Str. thermophilus* made the best starter, since there was continuous growth of one organism or the other. *Str. lactis* eventually predominated in the cheese. Lactobacilli slowly increased in number during later stages of ripening. Pasteurization of the milk was an effective control of gassiness.

In Stilton cheese the bacteria rapidly increased in numbers during the first few days, then steadily declined until there was a temporary rise at the time of blueing. Streptococci predominated at first, but later the number of rods increased. Sections of cheese showed these rod forms to be particularly abundant in and around the veins in which the mould was growing. There was evidence that some of the late-appearing rods were proteolytic (Hiscox, Harrison & Wolf(144)). The work of Dattilo-Rubbo on the taxonomy of the moulds of Stilton and other blue-veined cheese was cited in a previous review(145). Bryant & Hammer(146) traced the cause of a sudden failure of mould growth in blue cheese (Roquefort type) to the use of a culture of an atypical strain of *P. roquefort*. Black discolorations may be caused by *Hormodendrum olivaceum*. *Bact. aerogenes* did not cause gas-holes in this type of cheese.

Two distinct, constantly occurring types of yeast were isolated by Guittonneau,

Keilling & Delaval(147) from the surface of Camembert cheese. Both were lactose-fermenters and tolerant to 10% salt concentrations. The type which readily fermented lactose did not actively attack lactic acid; the other type, a feeble lactose-fermenter, attacked lactic acid readily. This action, and their salt tolerance, made them peculiarly suited to grow in conjunction with lactic-acid bacteria on the surface of a dry-salted cheese. Similarly, salt-tolerant yeasts were found by Kelly & Marquardt(148) to be an integral part of the surface of Limburg cheese. The two predominant types of yeast grew over a wide range of acidity with a minimum at *p*H 3·5. *Bact. linens*, also salt tolerant, grew at a minimum *p*H of 5·85, which provides a reason for the sequence of the surface flora of this cheese. Work reviewed by these authors suggest that a similar sequence of changes occurs in the surface flora of Roquefort, Port du Salut and Tilsit cheese.

Kelly(149) found that even very small numbers of coliform organisms may cause gassiness in Limburg cheese, where conditions are very favourable for their growth. The fault could be controlled by pasteurization of the milk. Yale(150), Kara-Oglanov(151) and Karnahl(152) also report that pasteurization of the milk yielded cheese of better and more uniform quality. The method of manufacture may need some modification, and the degree of heating of the milk appeared to influence the ripening process.

Orla-Jensen(153, 154) has reviewed the researches on cheese ripening since the time of Duclaux. He holds the view that the ripening of hard cheese is an enzymatic process, and that living bacteria have no direct influence, since many of their enzymes are not liberated, and therefore cannot become active in the cheese, until after the death and autolysis of the organisms. Living bacteria may, however, play a part in the later stages of ripening of certain types of cheese, e.g. the propionic-acid bacteria in Emmental cheese. On the basis of this theory he has introduced a new type of cheese, rapidly maturing, presumably (no details are given) by means of enzymes.

The use of enzymes (notably lipase) to ensure the development of specific flavour constituents has been studied for various types of cheese. In blue-veined cheese the mould is usually a sufficiently active lipolytic agent to ensure the characteristic flavour, but in hard-pressed cheese such as Cheddar, the lipolytic agents are less well defined and of uncertain occurrence, although it appears that slight lipolysis is essential to the development of the true Cheddar flavour. Hammer(155) observed that very rapid hydrolysis produced rancidity but, normally, this gradually disappeared. His experiments suggested that this was due to the activities of certain micro-organisms which slowly attacked sodium butyrate. Other experiments of Lane & Hammer(156, 157, 158) indicate the importance of the natural lipase of milk. Destruction of the natural lipase by pasteurization resulted in loss of flavour in the cheese. The addition of lipolytic enzymes such as bovine mammary tissue to the pasteurized milk caused an earlier development of the Cheddar flavour. Davis(159) found that a characteristic strong flavour developed in cheese when small quantities of a commercial lipase were added to the milk. With the addition of larger quantities a definite rancidity was produced and the volatile acids markedly increased. Hlynka, Hood & Gibson(160, 161) showed that added lipase was in part inactivated by rennin and pepsin. That the flavour development is not entirely due to the natural lipase of the milk is suggested by experiments reported by Wolf(162), who noted lipolysis of butterfat in 2 months by suspensions of cells of *L. casei* which had been digested with trypsin for 24 hr. at 37° C., although there was no lipolysis in ordinary cultures even at the end of 7 months.

The lipolytic enzymes of the moulds of the blue-veined cheese appear to be effective even while the moulds are actively growing. Jensen(163, 164, 165) found that the lipolytic activity of twenty-seven strains of *Penicillium* varied considerably both in degree and selectivity, and the flavour of Blue cheese (Roquefort type) varied with the strain used in their manufacture. Hiscox, Harrison & Wolf(166, 167) have shown that cheese

of Cheddar, Stilton and Roquefort types are to be distinguished by the quantity and distribution of their volatile acids. Important changes occur in the quantity and distribution of the volatile acids at the various stages of ripening of Stilton cheese, especially during the period of active growth of the mould. Marquardt & Dahlberg (168, 169) stressed the need for more accurate determinations of the volatile acids of cheese for purposes of control and research. After consideration of other methods they adhered to the direct steam-distillation method into which they introduced modifications which should make for greater accuracy. Their researches seem to be mainly concerned with hard cheese of the Cheddar type.

On the basis of his findings that a high concentration of CO_2 was a greater factor than lack of O_2 (unless the concentration was very low) in the inhibition of the growth of moulds of the *P. roquefort* group, Golding (170, 171, 172) has evolved a ripening process for blue-veined cheese in which the cheese are maintained in an atmosphere of controlled CO_2 concentration.

The acidity of a cheese in the early stages of manufacture may influence the whole course of the ripening. Researches therefore seek to determine the most suitable *p*H at different stages of the cheese-making process. Sherwood (126) has shown that gas production ('slit-openness') in New Zealand Cheddar cheese is favoured by sweet conditions in the cheese. Dolby (173) used *p*H measurements to follow the effect of variations in the technique of cheese making. Starter affected the acidity more in the early stages than later, but he found it was important to ensure that the acidity at the time of drawing the whey suited the particular starter and milk supply. This controlled the amount of lactose left in the curd and therefore the rate of increase of acidity in the cheese. Suitable values for the *p*H at various stages of ripening of Emmental cheese have been suggested by Virtanen (174), and Kelly & Marquardt (148) have indicated how changes in the *p*H of Limburger cheese determined the sequence of yeasts and *Bact. linens* in the surface flora.

The addition of salt in moderate amounts is mainly to influence the texture of the cheese, but in high concentrations it inhibits the growth of all but the salt-tolerant organisms. Thus Lane & Hammer (175) found that preliminary slight salting of the curd of blue cheese gave a more open texture and better mould growth than when the cheese were merely dry-salted or soaked in brine. Burkey, Rogosa & Farrar (135) found that heavy salting inhibited the growth of spore-bearing bacteria causing bitterness in Swiss cheese. The high salt content of the outer layers of Limburger cheese inhibited the growth of most bacteria and so favoured the development of the salt-tolerant yeasts and *Bact. linens* (Kelly & Marquardt (148)).

Wilson, Hall & Johnson (176) showed that 34° F. was a safe ripening temperature for cheese of unknown quality, since defects were largely suppressed by this procedure, but such cheese also lacked the typical Cheddar flavour. This developed in cheese ripened at 50° F., but since faults also arise at this temperature it can only be used on a large scale for cheese made from milk of good quality. Other things being equal, summer-made cheese were of better flavour than winter-made (Marquardt (177)).

The text-book on *Cheese* by Van Slyke & Price (178) (revised edition) should be of use not only to students but also to practical cheese makers. Surveys of the manufacture of Cheddar cheese in Rhodesia (179) and the Philippines (180) and a review of the literature on the pasteurization of milk for the manufacture of continental varieties of cheese (181) have been published. Doane (182) has related his own experience in a cheese factory when exact scientific laboratory control was substituted for subjective tests.

Considerable attention has been directed towards the provision of satisfactory methods of packing which will allow the storage and marketing of small quantities of mature cheese. Several publications bearing on this subject are relevant to this review. Canning is in many ways satisfactory, but undesirable flavours and odours

are apt to develop. According to Price(183) canned cheese of excellent quality was obtained at 34° F., but at 60° F. the cheese developed strong flavours. Doane(184) has stated that the accumulation of volatile substances may be avoided by sealing the cans with a water seal so that only very small positive or negative pressures occur in the can. Flavours due to the mineral salts in the cheese were eliminated by adding water to the cheese milk, so leaching out extra minerals. The period during which gas was evolved from the cheese was reduced to less than 2 months, and thus the cans could be sealed at a much earlier stage. It has also been reported that vacuum sealing(185) and the addition of tablets of harmless chemicals placed in the cans to absorb the gas(186) would prevent bulging. Wilson(187) has described a method by which Cheddar cheese curd is pressed into special forms, sliced and sealed in valve-vented cans in which it is ripened and sold for sandwich making. Similarly, Swiss cheese may be cut in slices and sealed in cans for storage. Various cellophane, metal and waxed paper wrappings have also been tested for their usefulness in the storage of cut pieces of mature cheese. The great drawback to this procedure is the development of moulds on the cut surfaces, though sometimes defects due to gassy or fruity fermentations may also occur (Reichart & Downs(188)). Very promising results were obtained by Price(189) by wrapping the cheese tightly in cellophane or aluminium foil and dipping it in a hot flexible wax. These cheese kept several months without change of flavour and only occasional mould spoilage. Delitsch(190) has described a number of moulds, some useful, others harmful, frequently met with in the dairy industry. Vernon(191, 192) has shown that good ventilation and suitable paintwork reduced the number of mould spores in the air of dairies. Ultra-violet radiations were not satisfactory for purifying the air, since mould spores are resistant to ultra-violet rays. A humidity of 80–85% in the ripening rooms reduced mould growth on the surface of cheese. Prolonged irradiation of the cheese itself did not control mould growth (Price(189)).

The problem has also been attacked by treatment of the coats or cut surfaces of the cheese with a harmless fungistat. The fatty acids seem to be peculiarly suited for this purpose. This question has been studied by Hoffman, Schweitzer & Dalby(193), who found that propionic, butyric, valeric, caproic, oenanthic, caprylic and pelargonic acids have marked fungistatic properties at *p*H 5 or lower. Acetic and formic were much less inhibitory, while the acids capric to myristic had little effect below *p*H 5, but at lower acidities were very fungistatic. The authors discuss the value of the fat-soluble acids in the destruction of such organisms as the tubercle bacillus and mould spores which bear a fatty envelope and are not therefore readily attacked by water-soluble bactericidal or fungicidal substances, and suggest also that they have a dietary value. Numerous papers have appeared dealing with the use of propionic acid and its salts as a preventative of mould on cheese(194, 195, 196, 197, 198, 199, 200, 201). The general findings were that propionic acid was much more inhibitory than acetic acid, and that the calcium or sodium salts were less efficient than the acid (the effect of propionic acid was very much reduced above *p*H 5(193)). The substances were not fungicidal but retarded mould growth. The mould-free life of wedges of cheese was doubled when the pieces were immersed in 5% solution of propionic acid for 15 sec., heat sealed into cellophane bags and stored at 58–60° F. (humidity 90%). The longer the immersion period the longer was the period of inhibition(198), but the solution usually impaired the colour and flavour of the surfaces. On the whole, the flavour of cheese so treated was not objectionable. Propionic and other fatty acids and their salts have been incorporated, with some success, into cream, butter, cream cheese and other foodstuffs. The development of mould has been delayed, but sometimes the flavour has been impaired. The rinsing of churns with propionic-acid solutions reduced the yeast and mould count of butter, and the treatment of wrapping papers with this acid appeared to be effective in suppressing mould growth on butter.

Dearden(202) has pointed out the importance of excluding air by the close adhesion of cheese and wrapper if the growth of moulds on cream cheese is to be avoided. Benzoic acid and its salts and esters appear to be less useful and effective than the fatty acids in inhibiting mould growth(203), and diacetyl was found to be as effective as benzoic acid in preserving edible fats(204). The frozen pack method has been found by Golding & Morgan(205) to be satisfactory for the preservation of Neufchâtel and Gervais cream cheese. The production of specially fine Roquefort cheese by selection and present methods of cold storage is slow and expensive, but Moulin(206) suggests wrapping cheese, taken at a late stage of ripening, in tin foil and storing at 4–6° C. at 90% humidity for a month. In his experiments the rind became eatable, the cheese lost no weight, and were of good flavour.

(*e*) Butter

The realization of the importance of *Pseudomonas* spp. in the deterioration of dairy products, especially butter and cream, is evident from the number of papers dealing with these organisms. Most *Pseudomonas* spp. are proteolytic, many are lipolytic, some are pigment producing, and some psychrophilic, characters which make them peculiarly fitted to cause defects in butter and cream.

Garrison(207) isolated and studied nearly 500 cultures of fluorescent bacteria mainly from milk and milk products, nearly half of which yielded fluorescent bacteria. Although most were *Pseudomonas* spp., many of which produced flavour or colour defects in butter, only about half could be identified with species already described. The isolation of these organisms from dairy products was greatly facilitated by enrichment in litmus milk at 3° C. Castell & Garrard(208) found the oxidase test a useful technique for tracking down these organisms in dairy products.

A black discoloration of butter, described by White(209), was due to an organism to which he gave the name *Ps. nigrificans*. There were two interesting features about this organism. Although it would grow readily at temperatures up to 37° C. the black pigment was produced only at low temperatures (4° C. was very favourable). Also, sodium chloride (or a similar substance) was essential for growth, but a concentration of 5% sodium chloride was the maximum that would be tolerated. The fault was of frequent occurrence, and since it has also been described for other dominion butter it is probable that the infection is widespread but only becomes evident when the butter is held at temperatures of domestic cold storage.

Two other species that have received special attention are *Ps. putrefaciens* (*Achr. putrefaciens*) and *Ps. fragi*, both of which cause defects in butter, the former a putrid and cheesy flavour, the latter a 'May apple' odour. Elliker & Horrall(210) have noted the loss of butter aroma before the development of putrid flavour by *Ps. putrefaciens*. They showed that this organism rapidly destroyed diacetyl in butter. Work on the characters and distribution of these two organisms has been carried out at the Iowa Experimental Station(211, 212, 213, 214, 215, 216). The organisms were found at the farms, in water supplies, feed, bedding, soil, and utensils. *Ps. fragi* was found in winter, but rarely in summer, probably because of its psychrophilic character. Enrichment methods were necessary for isolation in each case. Equipment at the creamery may also be a source of infection, parts of the churn inaccessible without dismantling being especially dangerous.

Castell & Garrard(217) found *Ps. fragi* and *Ps. fluorescens* in a high proportion of samples of well waters from country districts of Ontario. Wolochow, Thornton & Hood(218), in a survey of water supplies of Alberta creameries, found that *Ps. fluorescens* was a very common contaminant. Inadequate protection of well heads and other defective measures for the care of the water supplies was noted. An earlier investigation by Linneboe(219) had shown that in some cases the water was infected with *Achr. putrefaciens* as it left the well, and the infection increased when the water was

stored in tanks. In others the organism was introduced at the creamery through faults in management, such as passing pure and infected supplies intermittently through the same pipes.

Parfitt & Brown(220) found a close relationship between high counts of proteolytic and lipolytic bacteria and keeping quality of butter. Pont(221) has pointed out how rarely a single sample gives a reliable figure for the plate count of a box of butter. Even in good butter there was great variation within the samples. A single plating may give a quite misleading figure. Wilson & Prucha(222) used agar plating methods to trace changes in the flora during the making and storage of butter. In the freshly made butter, acid-producers were present, but alkali-forming, peptonizing, and inert types were in higher proportions than in the pasteurized cream. Very little change occurred in the butter stored at 40° F. for 3 weeks, but at 65° F. the acid-producing bacteria were almost completely eliminated. Peters(223) demonstrated moderate agreement between the counts of streak cultures on China blue lactose agar, and the quality of butter as judged by aroma, and flavour 2 days later. Yeasts and moulds, which did not affect the grading of the butter, tended to lessen the agreement. Olson(224) found that the bacterial count of butter decreased during storage at −25° C., but when unsalted butter was transferred to room temperature it deteriorated more rapidly than fresh samples held at room temperature.

Fragments of mould mycelia may frequently be found in butter. Adams & Parfitt(225) found that their number was determined largely by the degree of contamination of the cream. The time and temperature of storage of cream were important factors, as was also the air supply. Agitation of the cream during the daily accumulations favoured mould growth. They traced no significant connexion between presence or absence of moulds and the quality of the butter, nor had variations in the method of manufacture much influence on the mould-mycelium content of butter. As Vandaveer & Wildman(226) point out, however, the amount of mould mycelium in the butter serves as a useful index of the quality of the cream from which it is made. This in turn reflects the conditions at the farm. Wildman(227) suggests that the addition of one or two drops of crystal-violet solution to the mixture of gum and butter after the butter is melted increases the visibility of the fragments of mycelia in the visible mould test.

The reworking of butter disturbs the original distribution of water droplets and may result in the infection of sterile droplets. Long & Hammer(228) have investigated this problem and have shown that reworking butter accelerates the growth of bacteria and decreases the *p*H of the serum. The net result is a loss in keeping quality and the earlier appearance of defects.

III. PASTEURIZATION AND OTHER PROCESSES

(*a*) Pasteurization

With the stimulus given to the H.T.S.T. process by the increasing official sanction in America and, recently, in Great Britain, a large number of papers of general and scientific interest come within the scope of this review. Official hesitation has been due to the knowledge that the absolute margins of safety are so small that the safety of the product depends upon the efficiency of the plant to an even greater extent than in the holder process in which a certain amount of manual control is possible, if not desirable. The skill of the engineers has produced instruments so finely adjusted that precision control of the high temperatures and short holding times can be assured. The energy that has been thrown into the engineering problems is indicated by the large number of patents granted in Great Britain alone for improvements in design of all parts of the necessary plant (heaters, holders and control apparatus). Some of

the new patents are designed for holder plants, but in the main they apply to machinery that will constitute the integral parts of H.T.S.T. plants.

With the licensing of H.T.S.T. plant for use in the treatment of milk for human consumption has come the opportunity for testing this process on a commercial scale, and comparing it with the long-established holder process. This opportunity came earlier in America than in Great Britain. The Chicago experiment, in which a number of plants, of four approved designs, were allowed to be run on a large scale, with certain provisos, gave valuable information. The plants were all carefully supervised, and the results therefore provide a fair estimate of the efficiency of pasteurization at 160° F. for 16 sec. This is an important point, and if the process is to be judged from the performance of the increasing number of plants that may be expected to come into operation in Great Britain care must be taken that only plants that are competently run at the specified temperature/time combination shall be used in the compilation of data.

From the results of American investigations it would appear that the H.T.S.T. process (160 or 161° F. for 16 sec.) is not as effective as the holder process in the reduction of the general flora of milk. There has therefore been an attempt to increase the destruction of bacteria, without further injury to the chemical and physical properties of the milk, by interposing a supplementary short holding period at a lower temperature. This idea was put forward some years ago by Mattick & Hiscox with the tentative suggestion of 5 min. holding at 145° F. to be interposed either before or after heating and holding at 160° F. for 15 sec. Laboratory tests and experimental runs on a plant erected for the purpose gave satisfactory results(1), but so far the method has had no commercial application. The Cherry-Burrell company has designed a plant embodying a somewhat similar idea which they have termed the Duo process. Mention was made of this in the last review, but at that time reports of its performance were not available for reference. In this process milk from the regenerator is heated to 157° F., cooled to 143° F., reheated to 161·5° F., held at this temperature for 16 sec. and then cooled. Burrell(2) claimed that this modification considerably improved the percentage reduction of bacteria and had no deleterious effect on the cream line, but Dotterrer(3) reported tests that gave no evidence of any advantage in the Duo process even with an additional holding time of 9·5 sec. at 157° F. Plants adapted for this process were amongst those used in the Chicago experiment.

Quite other motives seem to have prompted the modifications introduced into German plants. Much latitude in the choice of temperature/time combinations appears to be permitted, but the heat treatment is usually more drastic than is customary in America or Britain. The milk is frequently held at 71–75° C. (159·8–167° F.) for 42–44 sec. This holding time is sometimes shortened by resorting to a 'Stufen' or an 'overswinging' process, in which the heating takes place in more than one stage(4). There is a preliminary rapid heating and cooling before the holding at the specified temperature, but, unlike the Duo process, the milk is heated above this temperature and must be cooled before holding. For example, in one type of machine the milk is heated to 76·5° C. (169·9° F.), held at this temperature for 2 sec., then cooled to 72·5° C. (162° F.) in 5·5 sec., held at this temperature for 5·5 sec., then cooled(5). The whole process occupies 15·5 sec. as compared with 47 sec. required for the process without the 'overswinging'. It is claimed that this modification gives results equal to those given by 145° F. for 30 min., but figures are not yet at hand for quotation.

Modifications involving two heating periods may have advantages in performance and economy, but since the safety of all short-time processes must depend largely on the efficiency of the automatic controls, the necessity of precision control will be increased. Moreover, the problem of defining equivalent temperature/time combinations at high temperatures has not yet been satisfactorily solved even when only one

heating and holding period is involved. The difficulty is increased when more than one period has to be considered. Although it is essential that the milk shall not be underheated there is no virtue in overheating.

The current work on the H.T.S.T. process quoted in this review refers generally to the direct process involving one heating and holding period, but the Cherry-Burrell plant, adapted for the Duo process, was used in some of the phases of the Chicago experiment. This was a large-scale investigation undertaken to satisfy the Health Authorities that the equipment designed for the H.T.S.T. process could be relied upon to yield a safe milk supply. Trial installations of four types of plant (Electropure, Isotherm, Precision and York Plate) with the later addition of the Cherry-Burrell Duo-pasteurizer were studied. The phosphatase test was used to indicate the safety of the milk (destruction of pathogenic organisms). The demonstration of the safety of the process was a necessary preliminary to the sanctioning of the conversion of old plant or the installation of new for the H.T.S.T. process during the time required to overcome troubles that had arisen as the result of heavy thermophile infection of the pasteurized milk. The trials were satisfactory, and permission was granted to use the H.T.S.T. process for the pasteurization of market milk supplies(6). The temperature/time combination at first used was 160° F. for 15 sec. but was later raised to 161° F. for 16 sec.

Reports on the bacteriological aspects of this experiment have been given by Krueger(7), Dotterrer(8), Parfitt(9) and Workman(10). They demonstrated that the thermophiles did not increase in number during this process, a confirmation of the work of other investigators. The presence of thermoduric organisms was, however, a serious source of trouble. Fluctuations in count during the run were more marked than those experienced with the holder process, and were traced to varying numbers of thermoduric organisms in the raw milk of different supplies. The continuous-flow process does not allow of mixing such as occurs in the large bulks in the vats of the holder plant.

Exact control of heating temperatures and holding times is of vital importance in a process which allows so small an absolute margin of safety. Accurate records and precision control devices are essential. This is fundamentally an engineering problem, but it is so intimately connected with bacteriological questions that several recent papers on this subject must be included in this review. Those by Riley(11), Keller(12, 13), Rishoi(14), Button(15), Fuchs(16), Weist(17) and Frank(18) deal with the plant as a whole, i.e. with the position and type of temperature recorders, checking of holding time, thermostatic control, regulation of pump speed and flow-diversion valves in general. Others deal with specific points. Roger(19) describes a method of checking holding times by injecting salt solution at the holder inlet and observing the time of the first deflexion on a galvanometer at the holder outlet. Lambert(20) discusses the points of good and bad thermometers, their correct installation in the plant and standard methods of checking. Description of various types of flow diversion valves are given in trade journals(21, 22, 23).

England(24) has described a small electric-holder pasteurizer of 12 gal. capacity suitable for small dairies. The milk is heated by means of carbon electrodes suspended in the vat. The power is automatically shut off when the temperature reaches 144° F. and remains off until the temperature falls to 143·5° F. (It could of course be adjusted for other standards.)

As has been pointed out by Frank(18) and Fuchs(16), milk problems place an increasingly great responsibility on engineers and inspectors, an adequate number of whom should be specially trained to deal with them. Skilled plant operators too should be demanded, and Griffiths(25) gives an account of courses of instruction and type of examination on which permits are granted in Oakland, California.

The colony count is widely used in the control of pasteurized milk. For a large

number of samples it is rather a long process and requires much glassware. American workers have always favoured a microscopical count in milk testing, but with pasteurized milk the result may be obscured because non-viable organisms may still be stainable. If, however, the pasteurized milk is suitably incubated before examination, the bodies of all but the thermoduric (living) organisms are disintegrated. Hileman & Leber(26) used 7 hr. incubation at 37° C. and Mallmann, Bryan & Fox(27) 2 hr. at 58–60° C. with satisfactory results. Microscopical counts may be used with good effect in control work by indicating the sources of contamination and their relative importance (Ward(28)).

The introduction of tryptone-glucose-extract-milk agar as a plating medium in American schemes of milk testing so increased the colony count of pasteurized milk that difficulty was sometimes experienced in keeping the count within the legal limits. This fact has been commented on by most of the American investigators. Their interest in thermoduric organisms, which are the chief cause of these high counts, has thus been heightened. In all cases the presence of thermodurics has been traced to ill-kept farm equipment, especially milking machines. Thermoduric bacteria are also resistant to chlorine solutions, concentrations of 100 p.p.m. for 2 min. or 20 p.p.m. for 5 min. being necessary for the destruction of a non-sporing organism tested by Maack & Prucha(29).

Hileman, Leber & Speck(30) found that six of the seven species of micrococci frequently isolated by them from pasteurized milk were identical with those previously shown by Breed(31) to be present in the normal cows' udder. They therefore concluded that the thermoduric micrococci were derived from the milk itself, their ultimate source being the udder. On the other hand, Gibson & Abdel-Malek(32, 33) failed to find these micrococci in samples of aseptically drawn or certified milk after pasteurization at 63° C. for 30 min. In their experience carefully produced milk could be practically sterilized by this treatment. From moderately contaminated milk the survivors were chiefly corynebacteria, sometimes also a small number of micrococci. The latter survived in significant numbers only from milk produced with no special care and therefore exposed to external contamination.

It is obvious that the control of thermodurics in pasteurized milk must be at the farm; the plant is not the primary source of the trouble. The best test for deciding whether a high count in pasteurized milk is due to thermodurics is laboratory pasteurization of the sample (at 145° F. for 30 min.). Either the raw milk or the pasteurized milk itself may be used(34, 38). The number of thermoduric organisms can then be determined either by plating or microscopical counting. Many of the papers already quoted deal with the question of the field control of thermoduric organisms in the raw milk supplies. Meanwell(35) has stressed the importance of cooling milk at the farm. The resistance of some organisms is greatly increased if held for a few hours at a favourable temperature.

Several papers contribute to our knowledge of the flora of pasteurized milk. Hileman, Leber & Speck(30) found that after laboratory pasteurization at 143° F. for 35 min. a high percentage of the survivors were acid producers, but that alkali producers were also numerous when the milk was heated at 161° F. for 16 sec. in a plant operating the Duo process. They noted a lower percentage of streptococci and a higher percentage of micrococci in the milk from the plant. The most common species of micrococci were *M. candidus*, *M. epidermidis*, *M. luteus* and *M. varians*, which were also the predominant flora of dirty milking machines, and other farm utensils. Wainess & Parfitt(36) described a thermoduric organism, *Sarcina thermodurica*, isolated from milking machines and dairy utensils and resistant to both holder and high temperature-short time pasteurization. Provan & Rowlands(37, 38, 39), in examining the milk from forty-one plants (holder process) in England, found that the colony count of the pasteurized milk gave no definite indication of keeping quality

at 15·5° C. A reason for this seems to be given by the results of later work(40, 41) on the changes in the flora of pasteurized milk during storage at 15·5° C. They found that acid-producing bacteria were predominant in the fresh samples, but at the end of 48 hr. were superseded by alkali-producing and peptonizing bacteria which had been present in very small numbers in the fresh samples. After 72 hr. acid-producing bacteria, different from those in the fresh samples, were predominant: These were chiefly *Str. lactis*, which was found only in very small numbers in the fresh samples. Thus the final spoilage was due to organisms which were probably not significant in the original platings. The acid-producing bacteria of the early stages were micrococci and rods belonging to the genus *Corynebacterium*. Gibson & Abdel-Malek(32, 33) found that from certified milk pasteurized at 63° C. for 30 min. the surviving flora consisted almost entirely of corynebacteria. From tank milk too, corynebacteria were amongst the surviving flora, but there were also micrococci with a predominance of streptococci, most frequently *Str. thermophilus*.

According to the findings of Sherman and his co-workers(42, 43) pasteurization destroys bacteria which grow at low temperatures so that the keeping quality of pasteurized milk at 0° C. is greater than would be expected from the keeping quality of the raw milk and the reduction in total bacterial count. Inoculation of the pasteurized milk with minute amounts of raw milk decreased the keeping quality to that of the raw milk. The spoilage appeared to be due almost entirely to *Pseudomonas* spp. which will grow at low temperatures.

Very little new information about thermophiles has been published, although their detection has been included in most of the investigations. Macy(44) found that when pasteurized milk was stored at 40° F. for 24 hr. the counts of 73·6% of the samples had decreased, though usually the difference was not great. Provan & Rowlands(41) also report a progressive decrease in the number of the thermophiles on storage of the milk at low temperatures. Walts(45) has given a résumé of our present knowledge of thermophilic bacteria in pasteurized milk.

It is well established that the temperature/time combination chosen for the holder process allows an ample margin of safety for the destruction of pathogenic organisms. This is not yet established as competently for the temperature/time combinations, 160, 161 or 162° F. for 15 sec., chosen for the high temperature-short time process, mainly because of the difficulty of exactly reproducing with laboratory apparatus the rate of heating and the very short holding time. Yet it is important that the equivalents shall be determined for the different temperatures. Holland & Dahlberg(46, 47) have studied the effect of heating over the temperature range of 140–180° F. on the creaming ability, the destruction of phosphatase and *Bact. coli*, rennet coagulation, calcium phosphate and nitrogen partition of the milk. Their results indicated that 170° F. was peculiarly suitable for pasteurization, since at this temperature *Bact. coli* and phosphatase were destroyed instantaneously and even a holding period of 2·5 sec. did not affect the creaming ability. Prucha & Corbett(48, 49) studied the destruction of *Bact. coli* and milk phosphatase and the effect on creaming power and flavour over a temperature range of 143–162° F. for periods varying from 5 sec. to 35 min. Supplee & Jensen(50) studied the effect of heat treatment at temperatures between 162 and 205° F. for relatively short periods. Using an apparatus in which the milk was electrically heated in thin continuously flowing films, so efficiently that it was exposed to the lethal temperature range of 145–185° F. for periods of 0·8–0·9 sec., he indicated that under these conditions bacterial destruction was equal to that of ordinary pasteurization processes, with minimum loss of flavour and physical properties of the raw milk. Logarithmic curves were plotted by each investigator, and while they agree in demonstrating that the destructive effect on bacteria was not quite parallel to the effect on creaming power, flavour or inactivation of the phosphatase, the equivalents deduced from their figures were not identical.

This is scarcely surprising when the differences in heating methods are considered. Holland & Dahlberg used thin-walled, rectangular, tinned copper tanks of 240 ml. capacity. Prucha & Corbett used small thin-walled test-tubes containing 3 ml. of milk (160 ml. were used for creaming and flavour tests). Supplee & Jensen used electro-pure apparatus and later a flowing film unit in which heat was generated electrically within the milk layer. It seems obvious that a continuous-flow method will be necessary if the equivalents determined experimentally are to be applied to commercial plant.

A cheap but simple method of pasteurizing dairy by-products before return to the farm for stock feeding has been described by Mattick & Hoy(51). The heating was accomplished by the direct injection of steam into the product as it left the regenerative section of a plate pasteurizer. This caused a rapid rise in temperature which was maintained for about 3 sec. in a short 'holding' pipe. Their experiments showed that heating at 170° F. would be effective for the destruction of tubercle bacilli, but they recommend 175° F. in order to meet possible fluctuations in temperature. There would naturally be a dilution of the whey or similar products, but this would not be a serious drawback in animal feeding.

The pasteurization of dairy by-products before being brought to the premises of an attested herd is compulsory. A recent amendment (16 October 1941) allows the use of the H.T.S.T. process in Great Britain. Holding at temperature not below 162° F. for not less than 15 sec. is specified. This would entail modification in the plant described by Mattick & Hoy.

Steam injection has been used in American plants designed for the removal of flavours from cream. The cream may be raised to temperatures considerably above its boiling-point by mixing the steam and cream under pressure. Rapid cooling at pressures below normal (by evaporation) removes volatile constituents of the cream and destroys many of the bacteria. Roberts, Coulter & Combs(52) have shown that cream was practically sterilized by heating to 260° F. in an experimental laboratory plant. The butter made from it appeared to have no greater keeping quality, however, than that made from cream vat-pasteurized at 160° F.

(*b*) Other processes

Various treatments, other than pasteurization, which are in limited use for reducing the number of bacteria in, and prolonging the useful life of milk, have been reviewed by Demeter(53). Rogers(54) showed that heat-resistant spores in milk were rapidly destroyed by the hydrogen peroxide produced during ultra-violet irradiation. Concentration, temperature and *p*H were important. The rate of destruction of cells of *Bact. coli*, *Bact. prodigiosum* and spores of *B. subtilis* was proportional to the intensity of ultra-violet light of wave-length 2537 A over a wide range of intensities (Lea & Haines(55)). From the experiments of Rentschler, Nagy & Mouromseff(56) it appeared that the resistance of individual organisms to ultra-violet radiation differed widely at various stages of the life cycle. The application of high-voltage gradients (of the order of 8000 V.) was shown by Sandorf(57) to effect a certain amount of bacterial reduction, but the process was considerably less lethal than ordinary pasteurization.

(*c*) Canned and dried milk

No method has yet superseded canning and drying for prolonged storage of milk. The various processes have been outlined by Crossley(58), who also introduced a 'Purple milk' medium (containing 1% peptone and 0·01% brom-cresol purple) for the detection and identification of spore-bearing bacteria (anaerobic as well as aerobic) in canned food products. For the examination of vegetable and milk products

autoclaved meat or fish paste must be aseptically added to the medium (59). Prickett (60) used direct microscopical counts as well as plate counts in the study of the quality of dried milk. Lithium hydroxide was a useful diluent for material soluble with difficulty. Nichols has made detailed bacteriological studies of dried milk powder (61) and canned milk products (62). Plate counts of the dried milk powder showed wide variations from 1400 to 149,000,000 per g. at 37° C. and from 100 to 1,300,000 per g. at 55° C. Variations were noted from factory to factory, also from the same factory at different times. Storage of forty powders at room temperature for 1 year confirmed previous observations that there is a progressive but irregular decrease in bacterial count and activity. The bulk of the flora consisted of streptococci which resisted heating at 60° C. for 20 min. These were of the *viridans* type (*Str. thermophilus* dominant) or *Enterococcus* type (mainly *Str. durans* variants). The organisms growing at 55° C. appeared to be mainly *B. calidolactis*. Experiments indicated that storage under nitrogen reduced the rate of destruction of bacteria. Similarly, Schätzel & Clausen (63) found no advantage in storing whole milk powders under nitrogen or CO_2. Contrary to other workers' findings Nichols's samples of evaporated milk and cream from retail sources were all sterile, but the percentage sterility was slightly less for samples direct from the factories. One group of spoilage defects was due to non-sporing organisms which gained access to the cans through faulty sealing. Bitterness and thinning were caused by heat-resistant spore-forming bacteria. No defects caused by anaerobes were encountered. Later experiments (64) with strains of these aerobic spore-forming bacteria gave no evidence that their heat resistance was affected by variations in the fat percentage, and no conclusive evidence that variations in the *p*H over the range 7·0–5·95 had any significant effect.

As regards sweetened condensed milk Agenjo Cecilia (65) has described a fishy odour, the cause of which was not definitely established, and an odour and taste of glue which appeared to be due to a non-sporing rod for which the name *Tbm. mathiacolle* was suggested. Nichols (62) traced the formation of buttons to two strains of *Penicillium* and an *Actinomyces*.

IV. DETERGENTS AND DISINFECTANTS

The great value of detergents in all washing processes has led to the synthesis and appraisement of large numbers of compounds in recent years.

The question of the compounding and use of detergents has been discussed simply but scientifically by Liddiard (1). He gives an explanation of the chemical and physical processes which underlie 'fob' or lather formation, wetting, contact time necessary for killing at various temperatures and concentration of detergent, the effect of hydroxyl-ion concentration and buffer value, emulsification and adsorption of fat, indicating the way in which the various constituents promote or hinder these reactions. In a final summary he discusses the bottle-washing process stage by stage, showing how these scientific principles have been applied to practice. Johnson & Roland (2) have studied primarily the deposition of milk film on dairy equipment and methods of removing it. They found that when milk was circulated through stainless-steel tubes the deposit from milk containing air was cheese-like (thought to be due to dehydration and superheating of milk at the surface of bubbles) and unlike the continuous thin film (believed to be the precursor of milk stone) from de-aerated milk. They compared the efficiency of various alkalis in removing these deposits of milk solids and fat from metal and glass surfaces. The efficiency of various alkali detergents for the cleaning of bottles, plant and other dairy equipment has been studied for various mixtures, some of known composition, but many of them proprietary articles. A report from the Danish State Research Dairy (3) dealt mainly with plant treatment and the removal of milk stone, and special attention was paid

to the effect on metals. A technical bulletin of the Diversey Corporation(4) gives a table of combinations of concentration of NaOH, time of contact and temperature suitable for the cleaning and sterilizing of bottles. It states that many proprietary compounds are ineffective in the concentrations and temperatures recommended by the makers. They considered the free caustic test the best index of the germicidal power of the compound. Schnoll(5) has described an apparatus, which is a modification of the Wheatstone Bridge, by means of which the percentage of free caustic in several tanks may be measured without interruption.

The efficiency of the cleaning process is impaired if the plant is not properly operated. During a survey of the bacteriological condition of washed milk bottles Brannon(6) found that only 57% complied with the standard of one bacterium per ml. capacity. In his opinion 95% should meet the public health requirements if the machines were properly operated. Similarly, Plock & Fischer(7), after exhaustive tests at Kiel and Weihenstephan, stress that good results can be obtained only when attention is paid to every detail. They drew up a scheme which, if observed, would give uniformity of operation in churn-washing plants.

Mattick & Sharpe(8), in studying the germicidal properties of detergents, using *Bact. coli* and *B. subtilis* as test organisms, found that the germicidal velocity decreased sharply with fall of temperature. The *K* values of NaOH were little affected by small additions of Na_3PO_4, but large additions increased them. The added substance was a good detergent but a poor germicide. The presence of 0·1% milk considerably reduced the rate of disinfection. This protective action of milk has been noted and recorded by many other workers. Nottbohm(9), for instance, noted that the addition of 2% milk to water suspensions of *Br. abortus* and *Myco. tuberculosis*, generally increased the time of contact of various detergents and germicides required for the destruction of the bacteria. He found that in aqueous suspensions *Br. abortus* was less resistant than *Myco. tuberculosis*. With the alkaline preparations, the strength of the solution and not the *p*H was the determining factor; the efficiency of the chlorine preparations depended chiefly on the active chlorine content.

An important factor in the choice of detergents and disinfectants for use in dairy plant is the degree of the corrosive action. This has been an integral part of some of the studies already cited, notably the Danish State Research Dairy. Aluminium was the metal most readily attacked whether by alkaline, acid or chlorine preparations(10, 11). Parker(12) has shown that an acidified-steam rinse increased the destruction of bacteria, enhanced the detergency, was non-corrosive and prevented the formation of milk stone.

Baker, Harrison & Miller(13) found that all the cationic detergents were very effective inhibitors of bacterial metabolism (Gram-positive and Gram-negative organisms were equally sensitive), some even in concentrations of 1 : 30,000. Few of the anionic detergents were as effective, and they were, moreover, selective for Gram-positive organisms. The activity of both types was strongly influenced by the *p*H and was at the maximum in the range which favoured the presence of undissociated molecules—alkaline range for the cationic, acid for the anionic. The maximum inhibition was exerted by lauryl, myristyl and cetyl compounds. Bergeim(14) has pointed out the toxicity towards yeasts and *Bact. coli* of the undissociated molecules of the fatty acids, formic, acetic and butyric.

Work has been continued on various chlorine sterilizers. Prouty(15) has given a digest of present knowledge. Their corrosive action and stability have been studied by Haller, Grant & Babcock(10), who found that sodium hypochlorites and chloramine-T were corrosive to metals, especially aluminium. Chloramine-T was more stable than buffered or unbuffered hypochlorite and was quite stable in the dark refrigerator. The available chlorine was dissipated by contact with metal (very rapidly in the presence of milk), especially during the first 2 min.; udder washing

caused only moderate loss. On the other hand, Harrison & Mattick(16) found variable but often considerable loss of available chlorine during udder washing. The addition of ammonia to neutral chlorine solutions was found by Weber, Bender & Levine(17) to increase the contact time necessary to kill spores of *B. metiens*. The efficiency of chlorine solutions increased with the acidity (*p*H 5–10). The maximum efficiency of chloramine (1 p.p.m. ammonia for each 4 p.p.m. available Cl) was at *p*H 6–8, but with 3 p.p.m. ammonia to 4 p.p.m. Cl it was at *p*H 6. Chlorine was a more effective sterilizer than ammonia chlorine at acidities higher than *p*H 9·3. For more alkaline reactions ammonia chlorine was more effective. More evidence of the importance of the reaction when testing the efficiency of chlorine sterilizers is provided by the work of Scales & Kemp(18). With heavy aqueous suspensions of mixed organisms in flasks, complete sterilization was generally obtained in less than 5 min. using 50 p.p.m. available Cl as hypochlorite at *p*H 6 and 7, but at *p*H 11 viable organisms were still present after 15 min. For spraying glass-lined tanks solutions of 50 p.p.m. at *p*H 6 were as effective as solutions containing 250 p.p.m. at *p*H 10. The use of an alkyl aryl sulphonate in conjunction with hypochlorites gave excellent results. The substance was a strong surface depressant and was particularly effective against spores.

Most experimenters have used aqueous suspensions of organisms for tests of disinfectants, but Neave & Hoy(19) used surfaces of metal trays, infected by the deposition of bacteria in suspension in milk. Chlorine solutions were poured over the trays which were then mechanically agitated. Neither the amount of alkali nor the *p*H of the solution appeared to have much effect unless the *p*H exceeded 11. Agitation was the important factor, and it was necessary to keep the solution in constant motion for at least $\frac{1}{2}$ min. The germicidal rate increased with, but was not directly proportional to, the concentrations.

Chlorine preparations are being increasingly used in the cowshed for the disinfection of milking machines, cows' udders and the milkers' hands, and there are some papers bearing on this aspect of the problem. Hoy & Neave(19, 20) have pointed out that hypochlorites cannot be relied upon in practice to give the same degree of sterility as steam. These can only be effective on thoroughly clean, smooth surfaces, but are greatly superior to mere washing with weak detergents and may be safely used for short periods, especially if the utensils are periodically sterilized with steam or boiling water. Hoy & Neave found that when the utensils and milking machines at one particular farm were washed and treated with chlorine-soda solution for a continuous period of 9 weeks, good results were achieved only during the first week after steaming. The use of chlorine-soda solution at 110–120° F. did not appear to be more destructive to the rubber-teat cups than 2–5 min. steaming, but their bacteriological condition was often unsatisfactory. Harrison & Mattick(16) found that in the presence of 2% added milk a solution containing 200 p.p.m. available chlorine was sufficient to kill the organisms present in 20 sec., findings which agree with unpublished results of Stableforth and in general with those of Waugh, Elliker, Hilton & Bullard(21).

Harrison & Mattick found that, when hypochlorite was added to the water used in washing the udder of an infected cow in quantities sufficient to maintain the concentration at 250 p.p.m., an exposure time of 10 sec. was sufficient to give a reasonable margin of safety. The necessary time of immersion of the udder cloth was ensured by using two cloths alternately. They propose a routine for cow washing, treatment of teat cups, and hand washing which should prevent the transmission of mastitis organisms. Waugh *et al.*(21) found that when mastitis organisms were inoculated on the surface of teats they were not destroyed by dipping the teats in a solution containing 400 p.p.m. available Cl in the presence of skim milk.

Pohle & Stuart(22) have reported hand-washing experiments by which they illustrate the persistency of bacteria on the human skin, many individuals having a characteristic residual flora. The number removed by a standard washing routine

was remarkably constant for each individual but was varied considerably from person to person. The use of 70% alcohol as a germicide at one stage in the washing routine gave a sudden fall in the bacteria present in each of the following rinse waters. Petroleum ether, however, did not show this persistent effect. Harrison (23) has recovered Lancefield Group *B. streptococci* from the hands of milkers even in herds where infection with mastitis was very light. He did not, however, find them on the hands of people with other occupations. The organisms were recovered even after the hands had been washed and disinfected by the routine generally adopted in the cowshed. This, obviously, is inadequate to eliminate the danger of the transmission of mastitis organisms on the hands of milkers. Thorough scrubbing of the hands with a good detergent is advocated as a preliminary to disinfection. The use of a 'milking fat' containing an innocuous germicidal substance to be applied to the hands (not the teats) may be a palliative. The use of germicidal substances to be applied to the udder has been advocated and various ointments are on the market. Seelemann & Siemonsen (24) have examined some of these and have found many to be non-germicidal. Use of such preparations in a number of herds over long periods had caused no obvious reduction in the number of new udder infections.

The use of non-toxic germicides for the prevention of mould growth on milk products is discussed in the section on cheese.

There have been inevitable delays in the compilation of this review which even now lacks that completeness which we would wish. It has, however, not been possible to obtain timely access to many of the journals which were formerly used.

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